

Bacterial proteases: targets for diagnostics and therapy

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Abstract Proteases are essential for the proliferation and growth of bacteria, and are also known to contribute to bacterial virulence. This makes them interesting candidates as diagnostic and therapeutic targets for infectious diseases. In this review, the authors discuss the most recent developments and potential applications for bacterial proteases in the diagnosis and treatment of bacterial infections. Current and future bacterial protease targets are described and their limitations outlined.

Introduction

Proteases are one of the largest functional groups of proteins, with more than 4,000 members currently described [1]. They are involved in many processes, including homeostasis, the establishment of infection and nutrient acquisition. However, it is the importance of bacterial proteases in viability and pathogenicity that make bacterial proteases particularly interesting candidates for diagnostic and therapeutic purposes, not least because many of these enzymes are secreted into the surrounding micro-environment of the bacterium, making them accessible for detection. Indeed, several recent publications have shown that proteolytic activity can be detected using specific peptide substrates coupled to fluorogenic or chromogenic labels, and that such substrates could potentially

provide a rapid and simple technique for the detection of protease-secreting bacteria [2]. Further, the development of bacterial protease-related and simple detection and virulence monitoring devices could potentially allow rapid and cheap monitoring of patient-specific changes to bacterial virulence over time, possibly leading to personalised medical treatment regimens. Finally, research into bacterial proteases and their substrates will allow the development of novel protease-inhibiting compounds that could potentially be used to limit the action of destructive proteases secreted by bacteria during clinical infections [3, 4].

Bacterial proteases: mode of action

Proliferation and growth

Bacteria are known to utilise several different proteases that participate in the assembly and disassembly of the bacterial cell wall during the process of bacterial growth and cell division [5]. The pathways involved in these processes depend on both the shape and Gram type of the bacterium [6]; however, peptidoglycan (PGN) synthesis and hydrolysis is a process required by almost all bacteria for growth [5]. During turnover of the bacterial cell wall, several PGN-hydrolysing enzymes (autolysins) are produced (Fig. 1). Two classes of enzymes function to digest the glycan backbone; the *N*-acetylmuramidases and the *N*-acetylglucosaminidases. Another group of autolysins are the enzymes which degrade the bond between the glycan backbone and the stem peptide. These enzymes separate the sugar backbone from the PGN stem peptide and are members of the class of *N*-acetylmuramyl-L-alanine amidases. Further digestion of the PGN requires cleavage between the amino acids present in the PGN stem peptide by several (D- and L-amino acid recognising) carboxy- and endopeptidases [5]. Importantly,

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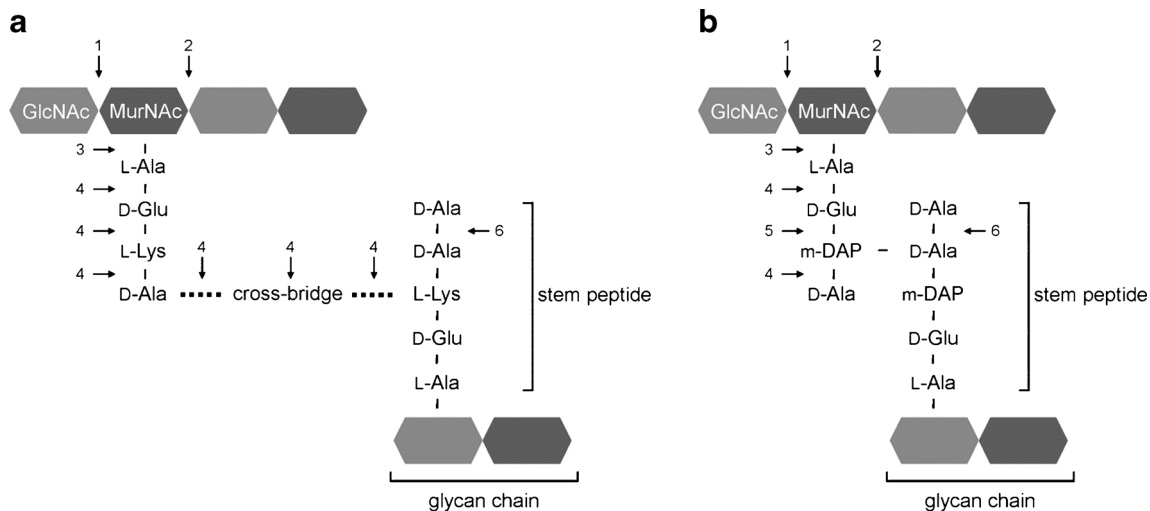


Fig. 1 Peptidoglycan hydrolases (autolysins) involved in peptidoglycan biosynthesis. **(a)** The Lys type and **(b)** the DAP type of peptidoglycan (PGN) are shown. The *arrows* indicate the cleavage sites of PGN hydrolytic proteases: 1=*N*-acetylmuramidase; 2=*N*-acetylglucosamidase; 3=

N-acetylmuramyl-L-alanine amidases; 4=other endopeptidases; 5=endopeptidases of the NlpC/p60 family, e.g. CwlS; and 6=carboxy- and transpeptidases, such as those of the penicillin-binding proteins (PBP) family [3]

because PGN is unique to bacteria, these D-amino acid processing autolysins are found exclusively in bacteria.

The most studied micro-organism in autolysin-related research is *Bacillus subtilis*. During the process of bacterial replication in *B. subtilis*, cell separation relies on the activity of the autolysins LytF, LytE and CwlS, all three of which are cysteine proteases and members of the NlpC/p60 endopeptidase family [7, 8]. Another autolysin, LytN, is a protease produced by *Staphylococcus aureus*, which functions as an amidase and endopeptidase during the synthesis of PGN [9]. The importance of autolysins in PGN synthesis has been demonstrated by the fact that deletion of these endopeptidases in *B. subtilis* or *S. aureus* results in cell growth defects and an altered growth morphology [10, 11].

Penicillin-binding proteins (PBPs) are a distinct group of autolysins which process D-amino acid bonds during PGN synthesis [12]. PBPs recognise and degrade the D-alanine–D-alanine bonds present in PGN and catalyse the terminal stages of PGN synthesis by creating cross-bridges between the stem peptides [1]. Though PBPs play an important role in cell morphology and viability, not all of the PBPs produced by bacteria are essential. For example, an *Escherichia coli* mutant lacking eight out of its 12 PBPs still remains viable, whereas deletion of the proteases PBP1a and PBP1b is lethal to the organism [13].

Virulence

Secreted bacterial proteases may degrade host-associated proteins, thereby playing a direct role in bacterial virulence (Table 1). Alternatively, bacterial proteases involved in growth and proliferation may actually contribute to bacterial virulence indirectly, by facilitating survival of the bacterium within the host environment. As examples, host proteins

involved in blood clot formation, such as fibrinogen, fibrin and coagulation factors, are a common target of bacterial proteases [14–17]. Further, the degradation of these proteins by bacterial proteases may lead to disease states such as disseminated intravascular coagulation (DIC), the formation of small blood clots in the blood vessel. This condition frequently occurs in patients with sepsis [18]. The role of bacterial proteases in DIC is supported by the findings of Komori et al., who observed that the fibrinogenolytic activity of the *Pseudomonas aeruginosa* LasA protease induces haemorrhagic tendency in mice [16]. In addition, several bacterial proteolytic virulence factors can target host connective tissue proteins. Infection of the periodontium, for example in infections related to the presence of *Porphyromonas gingivalis*, is characterised by destruction of the periodontal connective tissue as a result of host protein degradation by *P. gingivalis*-associated gingipains [19]. Additionally, the *P. aeruginosa*-specific proteases LasA and LasB can directly attack host tissue by degrading elastin, a component of the connective tissue [20, 21].

Many bacteria produce proteases involved in the “escape response” to various host defence mechanisms [22]. One of the defence systems targeted by bacterial proteases is the kallikrein/kinin contact activation system. When activated on the bacterial cell surface, the kallikrein/kinin contact activation system delivers antimicrobial peptides derived from kininogen and traps bacteria in the thrombus, while the major effector peptide, bradykinin, stimulates macrophages and induces an influx of neutrophils into the surrounding host tissue [23]. Cysteine proteases produced by *S. aureus* (staphopains SspA/B) and *P. gingivalis* (gingipains) degrade kininogen, which leads directly to the release of kinin [24]. The kinin released upon proteolytic degradation by these bacterial

Table 1 The most well-characterised bacterial proteolytic virulence factors and their (known) targets

Protease name	Catalytic type	Targets ^a	Micro-organism
Lethal factor (LF)	Metallo	MAPK kinase	<i>B. anthracis</i>
Staphopain A (SspA)	Cysteine	Kininogen	<i>S. aureus</i>
Staphopain B (SspB)	Cysteine	AMPs (cathelicidins) Kininogen Fibronectin Fibrinogen	<i>S. aureus</i>
Staphylolysin (LasA protease)	Metallo	Elastin Fibrinogen	<i>P. aeruginosa</i>
Elastase (LasB protease)	Metallo	Elastin AMPs (LL-37) Chemokines (RANTES, MCP-1)	<i>P. aeruginosa</i>
Gingipain R (Rgp)	Cysteine	Cytokines (IL-6, IFN- γ) Kininogen Coagulation factors (IX, X) AMPs (LL-37, histatin-5, dermaseptin, brevinin etc.) Chemokines (IL-8) Cytokines (IL-1 β , IL-6, IL-12, TNF- α , IFN- γ etc.)	<i>P. gingivalis</i>
Gingipain K (Kgp)	Cysteine	Neurotensin Haemoglobin Bradykinin Chemokines (IL-8) Cytokines (TNF- α) Synaptobrevin	<i>P. gingivalis</i>
Botulinum neurotoxins (BoNT)	Metallo		<i>C. botulinum</i>

^a Adapted from Potempa and Pike [22]

AMP antimicrobial peptide, MAPK mitogen-activated protein kinase

proteases induces vascular permeability and are assumed to promote an influx of plasma-containing nutrients into the site of infection. Additionally, invasion of the systemic circulation by *P. aeruginosa* can be facilitated by bradykinin generated by pseudomonal proteases [25].

Several bacterial proteases are able to inactivate antimicrobial peptides (AMPs) produced by the host. For example, the *P. aeruginosa* LasB protease and *P. gingivalis* gingipain R are able to degrade human AMP LL-37, an important component of the innate immune system [26, 27]. Other host proteins that are cleaved and inactivated by bacterial proteases include cytokines and chemokines, the communication signals of the innate and acquired immune system [28–32]. The interaction between bacteria and these host communication signals plays an important role in bacterial pathogenicity. Disturbance of the communication network by bacterial proteases may affect the clinical outcome of disease. Interestingly, it has been observed that degradation of human RANTES and MCP-1 by the *P. aeruginosa* LasB protease is accompanied by a loss of chemotactic activity, which suggests that *P. aeruginosa* may alter the relative amounts of critical immunomodulatory cytokines in the airway. This mechanism may contribute to the pathophysiology observed in *P. aeruginosa*-associated lung disease [32]. However, due to the complexity of the various

cytokine/chemokine functions and interactions, it is difficult to extrapolate the effect of bacterial proteases on these communication signals in vitro to the situation in infected tissue.

In summary, proteolytic bacterial virulence factors secreted at the site of infection can lead directly to host tissue destruction, haemorrhagic tendency and/or impaired clearance of the infection by the host immune system. Therefore, during infection of the host, the expression and secretion of proteases may provide bacteria with an evolutionary advantage over non-protease-secreting bacteria.

Bacterial proteases as diagnostic markers

Proteases are often secreted into the microbial environment and are, therefore, well suited for use as markers for the diagnosis of bacterial infections. Further, the relative ease of protease detection may enhance their potential as a diagnostic tool for detecting and monitoring bacterial infections.

Detection of proteolytic activity

Protease detection can be carried out by using different protein substrates and monitoring either disappearance of the

substrate or appearance of the cleavage product [33]. Natural substrates currently used in the measurement of in vitro bacterial protease activity include skimmed milk, casein, fibrin, elastin and gelatin. These substrates are added to bacterial solid growth media to detect overall protease activity. Cleavage of the substrate is indicated by the formation of clear halos around the colonies grown on the agar. A more specific approach to measure protease activity is the use of synthetic, colourimetric or fluorometric labelled peptide substrates which match the recognition sequence of the protease of interest [33, 34]. Depending on the labels attached to the peptide substrate, a read-out is performed using a spectrophotometer or fluorimeter. Most of the commercial kits available are based on the detection of proteolytic activity using peptide substrates. The use of peptide substrates enables proteolytic activity to be qualitatively and quantitatively measured in a relatively easy manner, and can also be adapted to high-throughput applications, e.g. for use in peptide-screening libraries [35–38].

Limitations of current detection approaches

The applicability of the above-mentioned substrates for the detection of bacterial proteolytic activity in clinical material has been explored by several research groups [38–40]. However, a persistent problem inherent in all of the existing techniques described is the non-specific cleavage of the substrates by unrelated bacterial and host proteases. This phenomenon seriously limits the detection of bacterial-specific proteolytic activity directly within clinical samples. Currently, this problem is circumvented by changing the read-out method or by the isolation of the microbial proteases of interest prior to their detection. For example, Boyer et al. developed a 24-amino-acid-containing FRET substrate for the detection of the proteolytic activity of anthrax lethal factor (LF), which is secreted by *Bacillus anthracis* [39]. Here, a confirmatory step using MALDI-TOF-based peptide detection was utilised to confirm if the cleavage pattern obtained was indeed related to LF-specific proteolytic cleavage. In another example, BonT (a *Clostridium botulinum* proteolytic virulence factor) was first pre-isolated by Dunning et al., using antibody-coated magnetic beads in order to isolate the BonT protease [40]. In general, the lack of specificity of peptide substrates, in combination with the relatively low sensitivity of existing methods for proteolytic enzyme detection, and labourious read-out mechanisms, has seriously limited the utilisation of protease detection in bacterial diagnostics and therapeutics.

Chirality: a proposed solution towards bacterial specificity

Objects are described as chiral if their mirror image cannot be superimposed on the original, with the mirror images of a

chiral molecule being referred to as optical isomers or enantiomers [41]. With the exception of glycine, all of the standard amino acids present in proteins possess an asymmetric carbon atom that can occur in two different configurations or optical isomers, named levorotary (L) and dextrorotary (D) [42]. While L-amino acids represent the vast majority of amino acids found in natural proteins, D-amino acids are far less common. In humans, D-amino-acid-containing proteins are rare, and are only found and processed in the brain [43]. However, as previously mentioned, a natural source in which large quantities of D-amino acids are found is the bacteria-specific PGN layer of the bacterial cell wall [3]. Therefore, D-amino acids are potentially useful in the design of FRET peptide substrates that could be used for the specific detection of bacterial proteolytic activity. In theory, the inclusion of D-amino acids into peptide substrates could lead to a decrease in non-specific cleavage by non-bacterial proteases, potentially enabling the direct measurement of bacterial proteolytic activity within clinical samples. Indeed, in a recent article, it was observed that short, D-amino-acid-containing peptide substrates tended to be cleaved by bacterial proteases involved in housekeeping processes and not by eukaryotic proteases or bacterial proteolytic virulence factors [44]. In addition, the presence of a D-amino acid in a peptide substrate abolished degradation of the substrate by human serum.

The actual applicability of D-amino-acid-containing peptide substrates in the diagnosis of bacterial infections was evaluated for *P. gingivalis*-related periodontitis. It was observed that the presence of D-amino acids in the peptide increased the specificity of the substrate for the detection of *P. gingivalis* in both saliva and patient-derived paper points [45, 46]. This research shows that the introduction of D-amino acids into peptide substrates may, indeed, be a valuable addition for improving the specificity of peptides for bacterial detection purposes.

Bacterial proteases as therapeutic targets

The growing prevalence of antibiotic-resistant bacteria underscores the need to discover novel treatment strategies and new bacterial targets for antimicrobial agents. One such novel target currently under investigation is the inhibition of bacterial proteases. The importance of bacterial proteases in bacterial viability and virulence makes them suitable targets for the development of novel antibacterial agents. Depending on the function of the protease targeted, the inhibition of bacterial proteolytic activity could lead to impaired growth, decreased virulence or an increase in susceptibility to antimicrobial agents. In fact, the effectiveness of therapies based on proteolytic inhibition has already been demonstrated in viral infections, e.g. in the treatment of hepatitis C and HIV-related infections. Infections related to these organisms are currently

treated by (competitive) protease inhibitors or drug combinations which include protease inhibitors [47, 48].

Antimicrobial

Many proteases produced by bacteria are essential for PGN biosynthesis. For this reason, proteases involved in PGN synthesis are potential targets for antimicrobial therapy. Previously, studies towards the development of antimicrobial therapies targeting PGN synthesis have predominantly focused on inhibition of the PBP cross-linking proteases [49–51]. These D-alanine-D-alanine carboxy peptidases are essential for bacterial growth and are present in all PGN-containing bacteria. The activity of PBPs is effectively inhibited by almost all currently used beta-lactam antibiotics. The effectiveness of PBP inhibitors in the treatment of bacterial infections has already been proven, as beta-lactam antibiotics are widely used to treat a broad spectrum of bacterial infections. However, novel PBP inhibitors are currently being developed in response to the rapid emergence of extended-spectrum beta-lactamase (ESBL) resistance [49, 52].

In addition to PBPs, other autolysins may also be potential targets for the development of protease-inhibiting antibiotics. In a recent publication by Singh et al., three new *E. coli* autolysins were identified [53]. Two of these proteases belong to the family of NlpC/p60 cysteine peptidases, while the other is a member of the lysostaphin family of proteins that cleave peptide cross-bridges. The researchers observed that a mutant lacking these autolysins underwent rapid lysis upon a shift to growth-restrictive conditions, indicating the potential of these proteases as antimicrobial targets. In addition, it was shown that the inhibition of cysteine protease activity leads to impaired bacterial growth [4, 54]. This is possibly due to a decrease in autolysin activity, similar to the activity of the NlpC/p60 protease.

Anti-virulence

In addition to the inhibition of bacterial viability, protease inhibitors can also be used to decrease bacterial virulence by targeting the proteolytic virulence factors secreted by bacteria. The inhibition of virulence was recently established as an antimicrobial strategy, with, for example, research currently ongoing into the development of anti-virulence proteins for LF, a toxin secreted by *B. anthracis* with protease activity [55–57]. Such anti-virulence treatment is necessary because of the limited efficacy of antibiotics in treating infections related to this organism. These efforts have led to the discovery of a diverse range of LF-inhibiting compounds, including an anthrax LF inhibitor found by Newman et al., which decreased the toxicity of LF to mouse macrophages and protected against LF-related activation of the Nlrp1b inflammasome in vitro [58]. In addition, studies on *P. aeruginosa* protease

LasB inhibitors are currently being performed [59, 60]. Recently, Cathcart et al. developed a potent inhibitor for the LasB protease and studied its ability to block virulence processes. It was demonstrated that the compound could completely block the action of LasB against protein targets, biofilm formation and immune modulation [60]. Thus, the inhibition of LasB activity may reduce pseudomonal pathogenicity and has the potential for combination with conventional antibiotic treatments. Another group of proteases which have been extensively investigated in research towards protease inhibitors are the *P. gingivalis*-specific gingipains [61, 62]. The compound DX-9065a was found to be a strong inhibitor of gingipain R (Rgp) activity and its pro-inflammatory effects in vitro [63]. In addition, DX-9065a partially inhibited the growth of *P. gingivalis*, which may be related to the fact that Rgp also degrades host proteins as a source of nutrition [64]. Therefore, as well as exerting anti-virulence effects, the inhibition of bacterial proteolytic virulence factors may also reduce bacterial viability.

Conclusions

The rapid detection and initiation of appropriate treatment for microbial infections is usually associated with an improved clinical outcome. Although the sensitivity of bacterial protease-based diagnosis is currently lower than that of genomic-based amplification techniques, e.g. polymerase chain reaction (PCR), D-amino-acid-containing peptide substrates have the potential to be developed for use as a supplemental, cheap and rapid diagnostic tool for bacteria-related infections. However, it should be noted that the ability of proteases to cleave a particular peptide substrate depends on a number of environmental factors, including pH, temperature and protease stimulators/inhibitors present in the surrounding environment. In addition, the sensitivity of proteolytic substrate-based detection technologies may depend on the variety and concentration of the proteases secreted by bacterial pathogens. In turn, this is affected by the availability of bacterial nutrients, the growth phase of the bacterium and the presence of other organisms in the micro-environment. However, the possibility of developing inexpensive diagnostics and novel protease inhibitors means that research will continue in this rapidly moving field of microbiology.

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References

- Rawlings ND, Barrett AJ, Bateman A (2012) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res* 40:D343–D350
- Loesche WJ, Giordano J, Hujuel PP (1990) The utility of the BANA test for monitoring anaerobic infections due to spirochetes (*Treponema denticola*) in periodontal disease. *J Dent Res* 69:1696–1702
- Drag M, Salvesen GS (2010) Emerging principles in protease-based drug discovery. *Nat Rev Drug Discov* 9:690–701
- Zindel S, Kaman WE, Fröls S et al (2013) The papain inhibitor (SPI) of *Streptomyces mobaraensis* inhibits bacterial cysteine proteases and is an antagonist of bacterial growth. *Antimicrob Agents Chemother* 57:3388–3391
- Humann J, Lenz LL (2009) Bacterial peptidoglycan degrading enzymes and their impact on host muropeptide detection. *J Innate Immun* 1:88–97
- Margolin W (2009) Sculpting the bacterial cell. *Curr Biol* 19:R812–R822
- Smith TJ, Blackman SA, Foster SJ (2000) Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology* 146:249–262
- Yamamoto H, Miyake Y, Hisaoka M, Kurosawa S, Sekiguchi J (2008) The major and minor wall teichoic acids prevent the sidewall localization of vegetative DL-endopeptidase LytF in *Bacillus subtilis*. *Mol Microbiol* 70:297–310
- Sugai M, Fujiwara T, Komatsuzawa H, Suginaka H (1998) Identification and molecular characterization of a gene homologous to *epr* (endopeptidase resistance gene) in *Staphylococcus aureus*. *Gene* 224:67–75
- Hashimoto M, Ooiwa S, Sekiguchi J (2012) Synthetic lethality of the *lytE* *cwlO* genotype in *Bacillus subtilis* is caused by lack of D,L-endopeptidase activity at the lateral cell wall. *J Bacteriol* 194:796–803
- Frankel MB, Schneewind O (2012) Determinants of murein hydrolase targeting to cross-wall of *Staphylococcus aureus* peptidoglycan. *J Biol Chem* 287:10460–10471
- Wise EM Jr, Park JT (1965) Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proc Natl Acad Sci USA* 54:75–81
- Denome SA, Elf PK, Henderson TA, Nelson DE, Young KD (1999) *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. *J Bacteriol* 181:3981–3993
- Imamura T, Potempa J, Tanase S, Travis J (1997) Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*. *J Biol Chem* 272:16062–16067
- Imamura T, Tanase S, Hamamoto T, Potempa J, Travis J (2001) Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from *Porphyromonas gingivalis*. *Biochem J* 353:325–331
- Komori Y, Nonogaki T, Nikai T (2001) Hemorrhagic activity and muscle damaging effect of *Pseudomonas aeruginosa* metalloproteinase (elastase). *Toxicol* 39:1327–1332
- Massimi I, Park E, Rice K, Muller-Esterl W, Sauder D, McGavin MJ (2002) Identification of a novel maturation mechanism and restricted substrate specificity for the SspB cysteine protease of *Staphylococcus aureus*. *J Biol Chem* 277:41770–41777
- Corrigan JJ Jr, Ray WL, May N (1968) Changes in the blood coagulation system associated with septicemia. *N Engl J Med* 279:851–856
- Potempa J, Banbula A, Travis J (2000) Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol* 2000 24:153–192
- Kessler E, Safrin M, Abrams WR, Rosenbloom J, Ohman DE (1997) Inhibitors and specificity of *Pseudomonas aeruginosa* LasA. *J Biol Chem* 272:9884–9889
- Moriyama K (1964) Production of elastase and proteinase by *Pseudomonas aeruginosa*. *J Bacteriol* 88:745–757
- Potempa J, Pike RN (2009) Corruption of innate immunity by bacterial proteases. *J Innate Immun* 1:70–87
- Frick IM, Björck L, Herwald H (2007) The dual role of the contact system in bacterial infectious disease. *Thromb Haemostasis* 98:497–502
- Imamura T, Tanase S, Szymid G, Kozik A, Travis J, Potempa J (2005) Induction of vascular leakage through release of bradykinin and a novel kinin by cysteine proteinases from *Staphylococcus aureus*. *J Exp Med* 201:1669–1676
- Sakata Y, Akaike T, Suga M, Ijiri S, Ando M, Maeda H (1996) Bradykinin generation triggered by *Pseudomonas* proteases facilitates invasion of the systemic circulation by *Pseudomonas aeruginosa*. *Microbiol Immunol* 40:415–423
- Schmidtchen A, Frick IM, Andersson E, Tapper H, Björck L (2002) Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* 46:157–168
- Carlisle MD, Srikantha RN, Brogden KA (2009) Degradation of human alpha- and beta-defensins by culture supernatants of *Porphyromonas gingivalis* strain 381. *J Innate Immun* 1:118–122
- Leidal KG, Munson KL, Johnson MC, Denning GM (2003) Metalloproteinases from *Pseudomonas aeruginosa* degrade human RANTES, MCP-1, and ENA-78. *J Interferon Cytokine Res* 23:307–318
- Matheson NR, Potempa J, Travis J (2006) Interaction of a novel form of *Pseudomonas aeruginosa* alkaline protease (aeruginolysin) with interleukin-6 and interleukin-8. *Biol Chem* 387:911–915
- Baba A, Kadowaki T, Asao T, Yamamoto K (2002) Roles for Arg- and Lys-gingipains in the disruption of cytokine responses and loss of viability of human endothelial cells by *Porphyromonas gingivalis* infection. *Biol Chem* 383:1223–1230
- Sheets SM, Robles-Price AG, McKenzie RM, Casiano CA, Fletcher HM (2008) Gingipain-dependent interactions with the host are important for survival of *Porphyromonas gingivalis*. *Front Biosci* 13:3215–3238
- Mikolajczyk-Pawlinska J, Travis J, Potempa J (1998) Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease. *FEBS Lett* 440:282–286
- Kasana RC, Salwan R, Yadav SK (2011) Microbial proteases: detection, production, and genetic improvement. *Crit Rev Microbiol* 37:262–276
- Matayoshi ED, Wang GT, Krafft GA, Erickson J (1990) Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* 247:954–958
- Oliveira LC, Silva VO, Okamoto DN et al (2012) Internally quenched fluorescent peptide libraries with randomized sequences designed to detect endopeptidases. *Anal Biochem* 421:299–307
- Ekici OD, Zhu J, Wah Chung IY, Paetzel M, Dalbey RE, Pei D (2009) Profiling the substrate specificity of viral protease VP4 by a FRET-based peptide library approach. *Biochemistry* 48:5753–5759
- Schaal R, Kupfahl C, Buchheidt D, Neumaier M, Findeisen P (2007) Systematic identification of substrates for profiling of secreted proteases from *Aspergillus* species. *J Microbiol Methods* 71:93–100
- Wildeboer D, Hill KE, Jeganathan F et al (2012) Specific protease activity indicates the degree of *Pseudomonas aeruginosa* infection in chronic infected wounds. *Eur J Clin Microbiol Infect Dis* 31:2183–9
- Boyer AE, Quinn CP, Woolfitt AR et al (2007) Detection and quantification of anthrax lethal factor in serum by mass spectrometry. *Anal Chem* 79:8463–8470
- Dunning FM, Ruge DR, Piazza TM, Stanker LH, Zeytin FN, Tucker WC (2012) Detection of botulinum neurotoxin serotype A, B, and F

- proteolytic activity in complex matrices with picomolar to femtomolar sensitivity. *Appl Environ Microbiol* 78:7687–7697
41. Cava F, Lam H, de Pedro MA, Waldor MK (2011) Emerging knowledge of regulatory roles of D-amino acids in bacteria. *Cell Mol Life Sci* 68:817–831
 42. Meierhenrich UJ (2008) Amino acids and the asymmetry of life. Springer, Heidelberg, Berlin, New York
 43. Ohide H, Miyoshi Y, Maruyama R, Hamase K, Konno R (2011) D-Amino acid metabolism in mammals: biosynthesis, degradation and analytical aspects of the metabolic study. *J Chromatogr B Analyt Technol Biomed Life Sci* 879:3162–3168
 44. Kaman WE, Voskamp-Visser I, de Jongh DM et al (2013) Evaluation of a D-amino-acid-containing fluorescence resonance energy transfer peptide library for profiling prokaryotic proteases. *Anal Biochem* 441:38–43
 45. Galassi F, Kaman WE, Anssari Moin D et al (2012) Comparing culture, real-time PCR and fluorescence resonance energy transfer technology for detection of *Porphyromonas gingivalis* in patients with or without peri-implant infections. *J Periodontol Res* 47:616–625
 46. Kaman WE, Galassi F, de Soet JJ et al (2012) Highly specific protease-based approach for detection of *Porphyromonas gingivalis* in diagnosis of periodontitis. *J Clin Microbiol* 50:104–112
 47. Matthews SJ, Lancaster JW (2012) Telaprevir: a hepatitis C NS3/4A protease inhibitor. *Clin Ther* 34:1857–1882
 48. Brower ET, Bacha UM, Kawasaki Y, Freire E (2008) Inhibition of HIV-2 protease by HIV-1 protease inhibitors in clinical use. *Chem Biol Drug Des* 71:298–305
 49. Contreras-Martel C, Amoroso A, Woon EC et al (2011) Structure-guided design of cell wall biosynthesis inhibitors that overcome beta-lactam resistance in *Staphylococcus aureus* (MRSA). *ACS Chem Biol* 6:943–951
 50. Lee M, Heseck D, Suvorov M, Lee W, Vakulenko S, Mobashery S (2003) A mechanism-based inhibitor targeting the DD-transpeptidase activity of bacterial penicillin-binding proteins. *J Am Chem Soc* 125:16322–16326
 51. Turk S, Verlaine O, Gerards T et al (2011) New noncovalent inhibitors of penicillin-binding proteins from penicillin-resistant bacteria. *PLoS One* 6:e19418
 52. Zervosen A, Sauvage E, Frère JM, Charlier P, Luxen A (2012) Development of new drugs for an old target: the penicillin binding proteins. *Molecules* 17:12478–12505
 53. Singh SK, SaiSree L, Amrutha RN, Reddy M (2012) Three redundant murein endopeptidases catalyze an essential cleavage step in peptidoglycan synthesis of *Escherichia coli* K12. *Mol Microbiol* 86:1036–1051
 54. Björck L, Akesson P, Bohus M et al (1989) Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. *Nature* 337:385–386
 55. Bannwarth L, Goldberg AB, Chen C, Turk BE (2012) Identification of exosite-targeting inhibitors of anthrax lethal factor by high-throughput screening. *Chem Biol* 19:875–882
 56. Park HC, Sung SR, Lim SM, Lee JS, Kim SK, Yoon MY (2012) Proteolytic assay-based screening identifies a potent inhibitor of anthrax lethal factor. *Microb Pathog* 53:109–112
 57. Turk BE (2008) Discovery and development of anthrax lethal factor metalloproteinase inhibitors. *Curr Pharm Biotechnol* 9:24–33
 58. Newman ZL, Sirianni N, Mawhinney C et al (2011) Aurano-fin protects against anthrax lethal toxin-induced activation of the Nlrp1b inflammasome. *Antimicrob Agents Chemother* 55:1028–1035
 59. Cathcart GR, Gilmore BF, Greer B, Harriott P, Walker B (2009) Inhibitor profiling of the *Pseudomonas aeruginosa* virulence factor LasB using N-alpha mercaptoamide template-based inhibitors. *Bioorg Med Chem Lett* 19:6230–6232
 60. Cathcart GR, Quinn D, Greer B et al (2011) Novel inhibitors of the *Pseudomonas aeruginosa* virulence factor LasB: a potential therapeutic approach for the attenuation of virulence mechanisms in pseudomonas infection. *Antimicrob Agents Chemother* 55:2670–2678
 61. Kadowaki T, Yamamoto K (2003) Suppression of virulence of *Porphyromonas gingivalis* by potent inhibitors specific for gingipains. *Curr Protein Pept Sci* 4:451–458
 62. Bodet C, Piché M, Chandad F, Grenier D (2006) Inhibition of periodontopathogen-derived proteolytic enzymes by a high-molecular-weight fraction isolated from cranberry. *J Antimicrob Chemother* 57:685–690
 63. Matsushita K, Imamura T, Tomikawa M, Tancharoen S, Tatsuyama S, Maruyama I (2006) DX-9065a inhibits proinflammatory events induced by gingipains and factor Xa. *J Periodontol Res* 41:148–156
 64. Matsushita K, Imamura T, Tancharoen S et al (2006) Selective inhibition of *Porphyromonas gingivalis* growth by a factor Xa inhibitor, DX-9065a. *J Periodontol Res* 41:171–176