Chirality: The Key to Specific Bacterial Protease-Based Diagnosis?

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Chirality:
The Key to Specific Bacterial Protease-Based Diagnosis?

Chiraliteit:

de sleutel tot bacterie-specifieke

protease-gebaseerde diagnostiek?

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It`s all about cleavage!

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Amino acids

	Amino acid	Code	Charge at pH 6.0-7.0	Properties
A	Alanine	Ala	Uncharged	Hydrophobic
C	Cysteine	Cys	Uncharged	Hydrophobic
D	Aspartic acid	Asp	Negative	Hydrophilic
E	Glutamic acid	Glu	Negative	Hydrophilic
F	Phenylalanine	Phe	Uncharged	Hydrophobic, aromatic
G	Glycine	Gly	Uncharged	
H	Histidine	His	Positive	Hydrophilic, aromatic
I	Isoleucine	Ile	Uncharged	Hydrophobic
K	Lysine	Lys	Positive	Hydrophilic
L	Leucine	Leu	Uncharged	Hydrophobic
M	Methionine	Met	Uncharged	Hydrophobic
N	Asparagine	Asn	Uncharged	Hydrophilic
P	Proline	Pro	Uncharged	Hydrophobic
Q	Glutamine	Gln	Uncharged	Hydrophilic
R	Arginine	Arg	Positive	Hydrophilic
S	Serine	Ser	Uncharged	Hydrophilic
T	Threonine	Thr	Uncharged	Hydrophilic
V	Valine	Val	Uncharged	Hydrophobic
W	Tryptophan	Trp	Uncharged	Hydrophobic
Y	Tyrosine	Tyr	Uncharged	Hydrophobic, aromatic

Introduction

Chapter 1

General introduction, aim and outline of the thesis

Submitted for publication

Introduction

Proteases are one of the largest functional groups of proteins, with more than 560 members described [1]. The fact that proteases hydrolyse one of the most important chemical bonds present in biomolecules i.e., peptide bonds, means that proteases exert crucial functions in a wide range of organisms, including bacteria. Proteases are essential for the proliferation and growth of bacteria, and are also known to contribute to bacterial virulence. Their importance in bacterial viability and pathogenicity make bacterial proteases ideal candidate diagnostic and therapeutic targets for infectious diseases. In addition, proteolytic activity can be easily detected using specific peptide substrates coupled to fluorogenic labels; such substrates could potentially provide a rapid and simple diagnostic technology for the detection of protease-secreting bacteria. Moreover, diagnosis using bacterial proteases provides information on bacterial viability and can be used to monitor changes in bacterial virulence over time. Finally, the development and use of bacterial substrates allows evaluation of the effect of protease inhibitors on bacterial protease activity and bacterial growth.

Bacterial proteases: mode of action

Proliferation and growth

Bacteria employ several proteases that participate in assembly and disassembly of the bacterial cell wall during the processes of bacterial growth and cell division [2-4]. The pathways involved in these processes depend on both the shape and Gram-type of a bacterium [5]. One component of the growth machinery is present in nearly all bacteria: peptidoglycan (PGN) synthesis and hydrolysis [4,6,7]. PGN is a polymer consisting of sugars and amino acids that forms the cell wall outside the plasma membrane. This structure provides strength to the bacteria, and serves to counteract the osmotic pressure of the cytoplasm. Additionally, PGN plays an important role in cell division during bacterial cell reproduction. The PGN glycan chain consists of two alternating amino sugars: *N*-acetylglucosamine (GlcNAc or NAG) and *N*-acetylmuramic acid (MurNAc or NAM). A short (4- to 5-residue) D-amino acid rich peptide chain (stem peptide) is attached to MurNAc. In *Escherichia coli*, this peptide chain contains L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine, whereas the stem peptide of *Staphylococcus aureus* consists of D-glutamine, L-lysine, and D-alanine, with a penta-glycine cross-bridge. During turnover of the cell wall, the PGN stem peptide is hydrolysed by

the D-amino acid-recognising autolysins (Figure 1). These autolysins are present in the bacterial cytoplasm of all PGN-containing bacteria. As PGN is unique to bacteria, D-amino acid processing autolysins are found exclusively in bacteria.

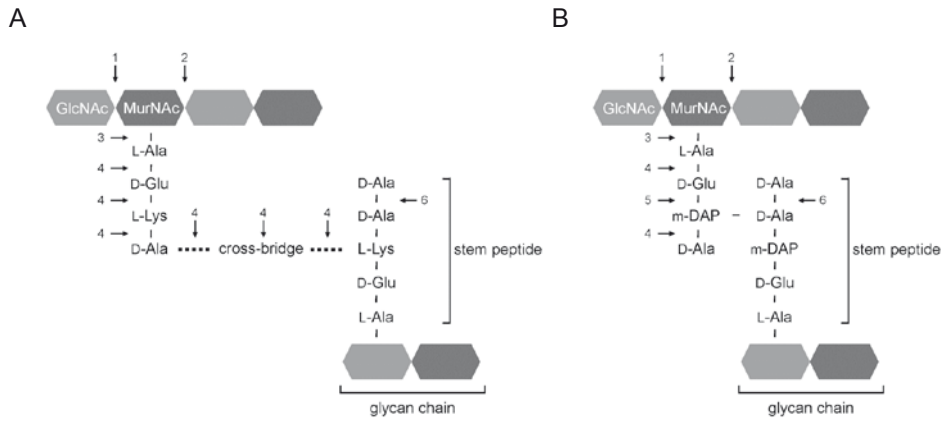

Figure 1. Peptidoglycan hydrolases (autolysins) involved in peptidoglycan biosynthesis. Both the Lys-type (A) and the DAP-type (B) of peptidoglycan (PGN) are shown. The arrows indicate the cleavage sites of PGN hydrolytic proteases: 1 = N-acetylmuramidase; 2 = N-acetylglucosamidase; 3 = N-acetylmuramyl-L-alanine amidases; 4 = other endopeptidases; 5 = endopeptidases of the NlpC/p60 family, like CwlS; and 6 = carboxy- and trans-peptidases, such as those of the penicillin-binding proteins (PBP) family [4].

The most studied microorganism in autolysin-related research is *Bacillus subtilis*. During the process of bacterial replication in *B. subtilis*, cell separation depends on the activity of the autolysins LytF, LytE, and CwlS, all of which are cysteine proteases and members of the NlpC/p60 endopeptidase family [8,9]. Another example of such an autolysin is LytN, a protease produced by *S. aureus* which functions as an amidase and endopeptidase during the synthesis of PGN [10]. The essential role of the autolysins in PGN synthesis has been demonstrated by the fact that deletion of these endopeptidases in *B. subtilis* or *S. aureus* induces cell growth defects and an altered growth morphology [11,12].

Penicillin binding proteins (PBPs) are a distinct group of autolysins which process D-amino acid bonds during PGN synthesis [13]. 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; an *E. coli* mutant lacking eight out of its 12 PBPs remains viable, whereas deletion of the proteases PBP1a and PBP1b is lethal to the organism [14].

Virulence

Bacterial proteases involved in growth and proliferation indirectly contribute to bacterial pathogenicity by facilitating replication of the bacterium within the host. In addition, many bacteria secrete proteases that actually are able to degrade host-associated proteins, and therefore play a more direct role in bacterial virulence (Table 1). The host proteins involved in blood clot formation, such as fibrinogen, fibrin and coagulation factors, are a common target of bacterial proteases [15-18]. Degradation of these proteins by bacterial proteases suggests the possibility that bacterial proteases induce disseminated intravascular coagulation (DIC), a condition which occurs frequently in patients with sepsis [19]. The role of bacterial proteases in DIC is supported by findings of Komori et al., who observed that the fibrinogenolytic activity of the *Pseudomonas ae-*

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

Protease name	Catalytic type	Targets [a]	Microorganism
Lethal Factor (LF)	Metallo	MAPK kinase	*B. anthracis*
Staphopain A (SspA)	Cysteine	Kininogen	*S. aureus*
		AMPs (cathelicidins)	
Staphopain B (SspB)	Cysteine	Kininogen	*S. aureus*
		Fibronectin	
		Fibrinogen	
Staphylolysin (LasA protease)	Metallo	Elastin	*P. aeruginosa*
		Fibrinogen	
Elastase (LasB protease)	Metallo	Elastin	*P. aeruginosa*
		AMPs (LL-37)	
		Chemokines (RANTES, MCP-1)	
		Cytokines (IL-6, IFN-γ)	
Gingipain R (Rgp)	Cysteine	Kininogen	*P. gingivalis*
		Coagulation factors (IX, X)	
		AMPs (LL-37, histatin-5, dermaseptin, brevinin, etc.)	
		Chemokines (IL-8)	
		Cytokines (IL-1β, IL-6, IL-12, TNF-α, IFN-γ, etc.)	
Gingipain K (Kgp)	Cysteine	Neurotensin	*P. gingivalis*
		Hemoglobulin	
		Bradykinin	
		Chemokines (IL-8)	
		Cytokines (TNF-α)	

[a] AMP: antimicrobial peptide, MAPK: mitogen-activated protein kinase.

ruginosa LasA protease induces hemorrhagic tendency in mice [17]. In addition, several bacterial proteolytic virulence factors can target proteins present in host connective tissue. Infection of the periodontium, for example in infections related to *Porphyromonas gingivalis*, is characterized by destruction of the periodontal connective tissue as a result of host protein degradation by *P. gingivalis*-associated gingipains [20]. Additionally, the *P. aeruginosa*-specific proteases LasA and LasB can directly attack host tissue by degrading elastin, a component of the connective tissue [21,22].

Many bacteria produce proteases involved in the escape response to various host defence mechanisms [23]. One of the defence systems targeted by bacterial proteases is the kalikrein/kinin contact activation system. When activated on the bacterial cell surface, the kalikrein/kinin contact activation system delivers antimicrobial peptides derived from kininogen and entraps bacteria in the thrombus, while the major effector peptide bradykinin stimulates macrophages and induces an influx of neutrophils into the surrounding host tissue [24]. Cysteine proteases produced by *S. aureus* (staphopains SspA/B) and *P. gingivalis* (gingipains) degrade kininogen which leads directly to the release of kinin [25]. The kinins released upon degradation by these bacterial proteases induce 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 [26]. Moreover, 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 [27,28]. 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 [29-33]. 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. 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 *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 [29]. 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, hemorrhagic tendency, and/ or impaired clearance of the infection by the host immune system. Therefore, during infection of the host, proteases may provide protease-expressing bacteria with an evolutionary advantage over non-protease-producing bacteria.

Antimicrobial resistance

Bacteria possess a broad variety of mechanisms which enable them to become resistant to antimicrobial agents. One of these mechanisms involves the production of antibiotic-degrading proteases that generate resistance to penicillin-like antibiotics e.g., the beta-lactamases [34,35]. The penicillin-like antibiotics targeted by bacterial proteases include various groups of antibiotics e.g., penicillins, cephalosporins, and carbapenems [36,37]. A common feature of these penicillin-like antibiotics is the presence of a four atom ring in their structure: the beta-lactam ring. Resistant beta-lactamase-producing bacteria inactivate beta-lactam antibiotics by hydrolysing the beta-lactam ring, thereby inactivating the compound (Figure 2).

Another mechanism via which bacteria are able to gain resistance to antimicrobial agents is by reprogramming their cell wall synthesis. This process requires the production of adapted proteases that are specific to resistant bacteria and are able to bypass the normal mechanism of cell wall synthesis. One example of this process is the development

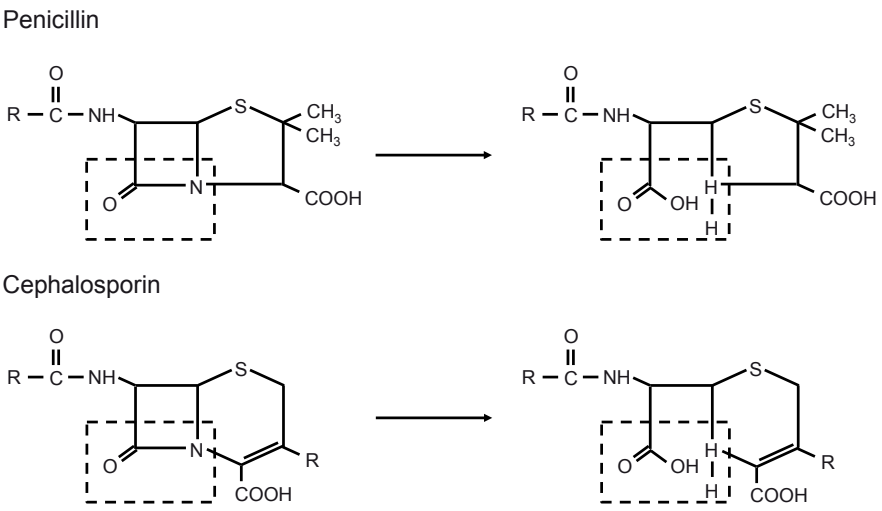

Figure 2. Target sites of the antibiotic-degrading beta-lactamases. Beta-lactamases inactivate penicillin (-like) antibiotics and cephalosporins by hydrolysing the beta-lactam ring present in these antimicrobial compounds.

of vancomycin resistance in *Enterococcus* spp.. *Enterococcus faecium* isolates which are resistant to vancomycin reprogram the termini of their PGN intermediates from D-alanyl-D-alanine termini to D-alanyl-D-lactate termini [38,39]. This change in the PGN terminus leads to a significant decrease in the affinity of vancomycin for PGN (Figure 3); examples of proteases involved in this process are the D-alanyl-D-alanine dipeptidases VanX and VanY [38,39].

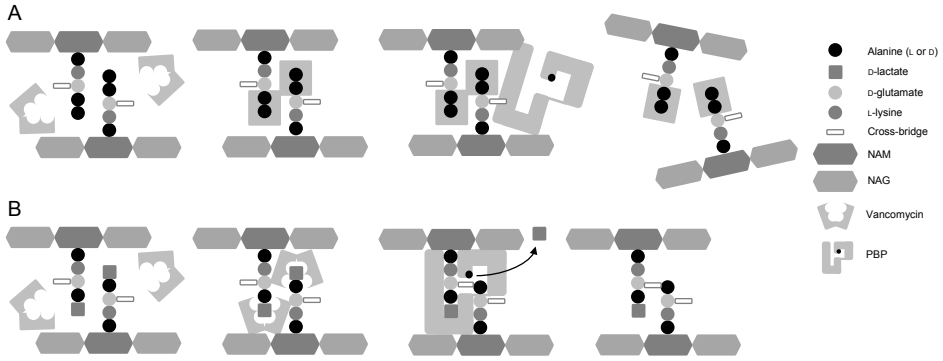

Figure 3. Mechanism of vancomycin action and resistance. Vancomycin recognises and binds to the two D-alanine residues present on the end of the PGN stem peptide. This prevents the stem peptide from interacting with the cell wall cross-linking protease, PBP. As a result, cross-bridges cannot be formed and the cell wall falls apart (A). In vancomycin-resistant bacteria, the last D-alanine residue has been replaced by a D-lactate residue; therefore, vancomycin is unable to bind to the PGN stem peptide and stable cross-bridges between the stem peptides can be formed (B).

Bacterial proteases as diagnostic markers

Bacteria produce many types of proteases, with large variations in their patterns of expression and function. These proteases are often secreted into the microbial environment and are therefore well suited for use as markers for the diagnosis of bacterial infections. Furthermore, the relative simplicity of detecting proteases may enhance their potential as a diagnostic tool.

Detection of proteolytic activity

To date, numerous methods have been developed for the detection of proteases, including gel electrophoresis and mass spectrometry [40]. However, neither of these methods can be easily applied to the quantification of protease activities, and they either require expensive equipment or have a low level of sensitivity. Enzyme-linked immunosorbent assay (ELISA)-based approaches offer a high flexibility, but have a restricted limit of

detection and are therefore less suitable for detecting proteases [41,42]. A rapid and simple alternative for the detection of protease activity is the use of fluorescence (or Förster) resonance energy transfer (FRET)-coupled peptide substrates. The use of FRET-peptide substrates to monitor protease activity was first described in 1990 by Matayoshi et al., who used the technique to determine HIV protease activity [43]. FRET-peptide substrates, in which the recognition and cleavage site of the protease of interest are present, are labelled with a fluorescent donor group and a quenching acceptor group. When the peptide is intact, and the distance between the donor and accepter group is < 10 nm, the donor group can transfer energy to the acceptor group according to the resonance mechanism discovered by the German scientist Dr. Theodor Förster. In this mechanism, the emission spectrum of the fluorophore overlaps with the absorption spectrum of the acceptor, which results in quenching of fluorescence. However, upon recognition and cleavage of the peptide bond between the acceptor/donor pair by the protease of interest, the distance between the donor and acceptor group increases, and as a consequence the donor group liberates fluorescence that can be detected in time using a fluorimeter (Figure 4).

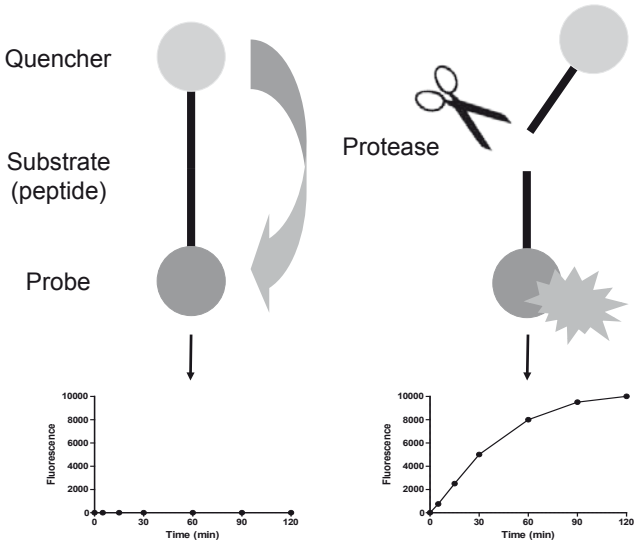

Figure 4. Principle of fluorescence resonance energy transfer (FRET). In an intact FRET-peptide substrate, the fluorescence of the probe (donor) is quenched by the acceptor group. When the peptide sequence of the substrate is recognised and cleaved by the protease of interest, the distance between the donor and acceptor pair increases. As a result, the acceptor molecule is unable to quench the fluorescence emitted by the probe, and an increase in the fluorescent signal correlating to the amount of proteolytic activity can be measured.

The use of FRET-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 such as, for instance, FRET-peptide screening libraries [44-46].

Limitations of current approaches

Protease activity is commonly monitored using FRET-peptide substrates. The applicability of these peptide substrates for the detection of bacterial proteolytic activity in clinical material has been explored by several research groups [47-50]. A persistent problem inherent in the existing techniques is the non-specific cleavage of the FRET-peptide substrates by unrelated proteases of eukaryotic origin. This hampers the detection of bacterial-specific proteolytic activity directly in clinical material. Currently, this problem is solved by changing the read-out method or by isolation of the microbial proteases prior to their detection. For example, Boyer et al. developed a 24-amino-acid-containing FRET-substrate for detection of the proteolytic activity of anthrax lethal factor (LF), which is secreted by *Bacillus anthracis* [49]. However, the chance of non-specific cleavage of the FRET-substrate by host-derived proteases greatly increases with the length of the substrate used. Therefore, 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 the case of BonT, a *Clostridium botulinum* proteolytic virulence factor, the problem of non-specific cleavage of the FRET-substrate was resolved by pre-isolation of the protease using antibody-coated magnetic beads, in order to prevent cleavage of the peptide substrate by the matrix in which the BonT protease was present [50]. In general, the lack of specificity of the cleavage of FRET-peptide substrates by bacterial proteases, in combination with the relatively low sensitivity of existing methods, currently seriously hampers the practical use of protease substrates in bacterial diagnostics.

Chirality: a proposed solution towards bacterial specificity

Objects are described as chiral if their mirror image cannot be superimposed on the original. The term chiral is derived from the Greek word for hand (χείρ), as the left and right hand provide a simple example of chirality. The mirror images of a chiral molecule are referred to as optical isomers or enantiomers [51]. 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) (Figure 5) [52]. While L-amino acids represent the vast majority of

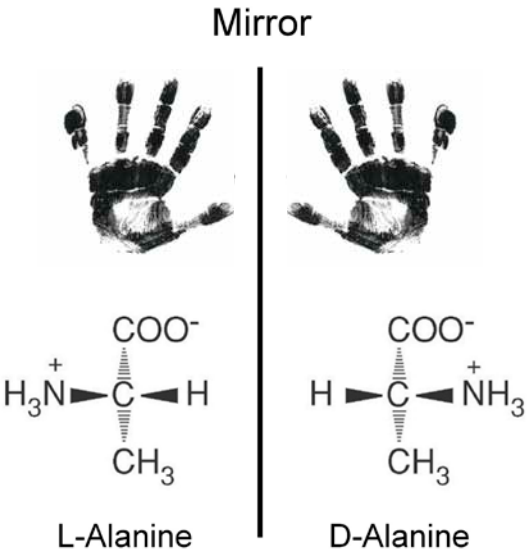

Figure 5. Chirality. Like human hands, L-alanine and D-alanine are mirror images of each other. While very similar in almost all characteristics, the mirror images of the left and right hand, or L-alanine and D-alanine, cannot be superimposed on each other.

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 [53]. A natural source in which large quantities of D-amino acids are found is the bacteria-specific PGN layer [4]. Therefore, D-amino acids may be useful in the design of FRET-peptide substrates that are to be used for the detection of bacterial proteolytic activity. The inclusion of "mirrored" D-amino acids in FRET-peptide substrates for the detection of bacterial proteolytic activity could lead to a decrease in the non-specific cleavage of the substrate by host (human) proteases. Therefore, the use of D-amino-acid-containing substrates may enable the direct measurement of bacterial proteolytic activity in clinical samples.

Bacterial proteases as therapeutic targets

The growing prevalence of antibiotic-resistant bacteria underscores the need to discover novel treatment strategies and 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 novel antibacterial agents. Depending on the function of the protease

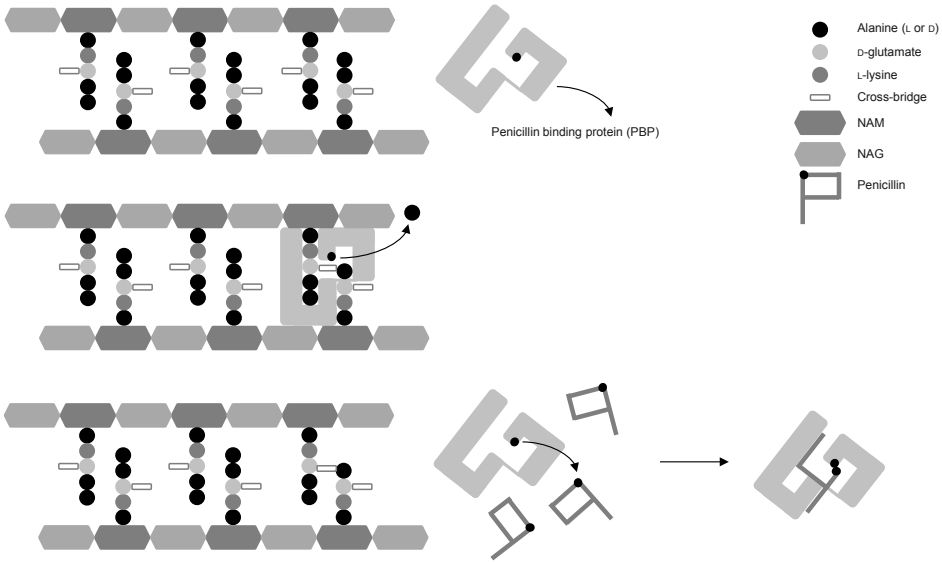

Figure 6. Mechanism of action and inhibition of penicillin binding proteins (PBPs). The PBP binds to the PGN stem peptide, removes a single D-alanine, and forms a cross-bridge between D-alanine and L-lysine. When the cross-bridge has been formed, the PBP dissociates from the bacterial cell wall. The activity of PBPs is inhibited by several antibiotics, including penicillin. Penicillin enters the active site of the PBP and reacts with the serine group (●) which is required for the proteolytic activity of the PBP. The beta-lactam ring of penicillin is irreversibly opened during the reaction with PBP. Penicillin remains covalently linked to the PBP which permanently blocks the active site.

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 [54,55].

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 [56-58]. 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 via the mechanism depicted in Figure 6. 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 [56,59].

Another approach by which beta-lactamase-related resistance can be overcome is the use of beta-lactamase inhibitors. Combinations of the beta-lactamase-based inhibitors clavulanic acid, sulbactam, or tazobactam, with beta-lactam antibiotics have already been successfully used as antimicrobial therapeutic strategies in the clinic [60]. However the above mentioned compounds possess narrow spectra of beta-lactamase inhibitory activities. As a result the activity of ESBLs and carbapenemases is not affected by these first-generation beta-lactam-based inhibitors. For this reason, several research groups have searched for novel beta-lactamase inhibitors [61]. For example, Aoki et al. investigated the potential of Ca-EDTA, an inhibitor of metalloprotease activity, in the treatment of carbapenem-resistant *P. aeruginosa* isolates. They observed that the susceptibility of resistant *P. aeruginosa* strains to imipenem increased in the presence of Ca-EDTA [62]. Furthermore, Livermore et al. developed a carbapenemase inhibitor, RPX7009, which successfully enhanced the susceptibility of carbapenem-resistant Enterobacteriaceae to the carbapenem antibiotic biapenem [63]. Thus, PBPs remain important targets for antimicrobial treatment, even though the continuing evolution of beta-lactam resistance requires the development of novel and more specific PBP and/or beta-lactamase inhibitors.

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 [64]. Two of these proteases belong to the family of NlpC/p60 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.

Recently, Mainardi et al. reported that the binding of beta-lactam antibiotics is not restricted to PBP transpeptidases. It has been shown in *E. faecium* that imipenem, a member of the carbapenem group of antibiotics, inhibits Ldt_{fm}, an autolysin known to cleave between the bond between L-lysine and D-alanine present in the peptidoglycan layer [65]. Ldt_{fm} is known to bypass the classical PBPs leading to high-level ampicillin resistance in *E. faecium*. Thus, combination therapy of imipenem-mediated Ldt_{fm} inhibition with ampicillin may lead to the eradication of resistant *E. faecium* isolates. The effectiveness of targeting autolysins to overcome antimicrobial resistance has also been observed

in vancomycin-resistant *E. faecium* isolates. Crowder et al. demonstrated that inhibition of the VanX dipeptidase increased the sensitivity of resistant *E. faecium* to vancomycin [66]. Therefore, the use of autolysin inhibitors in combination with antibiotics could thus potentially restore the effects of antimicrobial agents against multidrug-resistant *E. faecium* isolates.

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 *Bacillus anthracis* with protease activity [67-69]. 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* [70]. In addition, studies on *P. aeruginosa* protease LasB inhibitors are currently being performed [71,72]. 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 [72]. Thus, 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 [73,74]. The compound DX-9065a was found to be a strong inhibitor of gingipain R (Rgp) activity and its pro-inflammatory effects *in vitro* [75]. 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 [76]. Therefore, as well as exerting anti-virulence effects, the inhibition of bacterial proteolytic virulence factors may also reduce bacterial viability.

Aim and outline of the thesis

The overall aim of this study was to develop and evaluate D-amino-acid-containing FRET-peptide-substrates for the specific detection of bacterial protease activity. In particular, this work focused on the use of these substrates in bacterial diagnosis, prediction of bacterial virulence, and monitoring of protease inhibitor effectiveness.

Chapter 1 provides an overview of the applicability of bacterial proteases as targets for both bacterial diagnosis and antimicrobial treatment. The subsequent seven chapters are then divided into two main themes relating to the detection of bacterial proteolytic activity to diagnose infections (**Chapters 2,3,4 and 5**) and monitoring of the virulence and growth of bacterial pathogens (**Chapters 6 and 7**).

In **Chapter 2**, the applicability of a D-amino-acid-containing FRET-peptide library for the specific detection of bacterial proteases was evaluated. Screening this library with the periopathogen *Porphyromonas gingivalis* led to the discovery of five *P. gingivalis*-specific substrates. Next, the potential of these substrates to diagnose *P. gingivalis*-related periodontitis and identify the bacterial proteases involved in this process are described in **Chapter 3**. In **Chapter 4**, the specificity and sensitivity of the FRET-peptide substrates as a diagnostic tool was evaluated and compared to culture- and DNA-based methods.

The rapid and simple diagnosis of bacterial infections is essential when considering infections related to biological warfare agents, as such agents generally possess a high pathogenic potential and require accurate treatment to be initiated as soon as possible after infection. In this respect, in the study presented in **Chapter 5**, the development of a FRET-peptide substrate to detect *Bacillus anthracis* spores and to diagnose anthrax infections is described.

It has been known for many years that bacterial proteases play an important role in virulence and pathogenicity. In **Chapter 6**, the performance of a FRET-peptide substrate to predict the virulence potential of *Pseudomonas aeruginosa* clinical isolates was evaluated. As well as being involved in virulence, bacterial proteases are also involved in a large variety of housekeeping processes, reflecting the fact that proteases are essential for bacterial viability and growth. Therefore, in **Chapter 7,** the effect of a protease inhibitor isolated from *Streptomyces mobaraensis* on bacterial virulence and growth was studied.

Finally, **Chapter 8** summarises the research presented in this thesis and discusses the future perspectives and potential clinical applications of bacterial protease detection and inhibition.

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Diagnosis

Chapter 2

Evaluation of a D-amino-acid-containing fluorescence resonance energy transfer (FRET-) peptide library for profiling prokaryotic proteases

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Abstract

Bacterial proteases play an important role in a broad spectrum of processes, including colonization, proliferation and virulence. In this respect, bacterial proteases are potential biomarkers for bacterial diagnosis and targets for novel therapeutic protease inhibitors. To investigate these potential functions, the authors designed and used a protease substrate fluorescence resonance energy transfer (FRET)-library comprising 115 short D- and L-amino-acid-containing fluorogenic substrates as a tool to generate proteolytic profiles for a wide range of bacteria. Bacterial specificity of the D-amino acid substrates was confirmed using enzymes isolated from both eukaryotic and prokaryotic organisms. Interestingly, bacterial proteases that are known to be involved in housekeeping and nutrition, but not in virulence, were able to degrade substrates in which a D-amino acid was present. Using our FRET peptide library and culture supernatants from a total of 60 different bacterial species revealed novel, bacteria-specific, proteolytic profiles. Although in-species variation was observed for *Pseudomonas aeruginosa*, *Porphyromonas gingivalis* and *Staphylococcus aureus*. Overall, the specific characteristic of our substrate peptide library makes it a rapid tool to high-throughput screen for novel substrates to detect bacterial proteolytic activity.

Introduction

Proteolytic enzymes play a fundamental role in a large number of biological processes in both eukaryotes and prokaryotes. This includes nutrient acquisition, virulence and microbial defence [1–4]. The development of a simple, rapid, high-throughput screening methodology for bacteria-specific proteases would allow cost-effective screening of such bacterial proteases and their substrates, with potential applications in the development of bacterial diagnostics and novel therapeutic protease inhibitors, as well as identifying potential sources of enzymes useful in the pharmaceutical, food and biological industries.

Fluorescence resonance energy transfer (FRET) - peptides have previously been shown to be useful tools to monitor the rate and activity of proteolytic cleavage and for research into specific protease inhibitors [5,6]. For example, the protease inhibitor VX-950, which is currently used in the treatment of hepatitis C virus infections, was validated using FRET-peptides to monitor NS3 protease activity [7,8]. This FRET-peptide substrate approach was also used recently to examine the inhibitory effect of a novel *Streptomyces* Papain Inhibitor (SPI) against bacterial proteases [9].

In theory, bacterial proteases are also suitable as biomarkers for the identification of microorganisms in both environmental and clinical samples because proteases released into the surrounding environment are accessible for detection using FRET-substrates [10,11]. However, the substrates used in previous research could be potentially non-specifically cleaved by unrelated proteases of eukaryotic origin, requiring isolation of microbial proteases prior to their detection. For example, Boyer and co-workers developed a 24-amino-acid-containing substrate to detect anthrax lethal factor (LF) activity [12]. However, the length of the substrate increases the chances of non-specific substrate cleavage by unrelated bacteria and by host-derived proteases. In a separate publication, antibody coated magnetic beads needed to be used to purify BonT (a *Clostridium botulinum* proteolytic virulence factor) in order to prevent non-specific cleavage of the protease substrate by the matrix in which the protease was present [13]. This lack of specificity, in combination with relatively low sensitivity, has hampered the practical use of protease substrates for bacterial diagnostics.

Recently, the authors succeeded in enhancing the specificity of bacterial protease detection by exploiting the chiral nature of amino acids [10,14]. With the exception of glycine, all of the amino acids in human proteins possess an asymmetric carbon atom that can occur in two different configurations or optical isomers, named levorotary (L) and dextrorotary (D) [15]. Whereas L-amino acids represent the vast majority of amino acids found in natural proteins, D-amino acids are specifically produced and used

by bacteria and can be found, for example, as components of the bacterial cell wall [16]. Knowing that bacteria are able to process D-amino acids, we hypothesised that they must express proteases that recognise and hydrolyse D-amino-acid-containing substrates. Previous publications indeed showed that substituting an L-amino acid for its D-enantiomer abolished the degradation of FRET-peptide substrates by human proteases [10,14]. Further, the presence of D-amino acids appeared to significantly increase FRET-peptide substrate specificity in some bacterial species [10,14].

However, whether investigating the detection of novel proteases from bacteria, the characteristics of bacterial proteases, the potential use of proteases in bacterial diagnostics or novel therapeutic protease inhibitors, using a single protease substrate will lead to a serious reduction in the general specificity of any cleavage information obtained. Therefore, the use of multiple protease substrates, for example by developing a FRET peptide library to study bacterial protease activity, is highly recommended. Furthermore, the use of protease substrate-containing libraries allows for: i) the analysis of multiple proteases simultaneously without prior knowledge of substrate preference and ii) the generation of protease profiles or "fingerprints" [17–19]. Although previous groups have used FRET-substrate libraries comprising 5 or more L-orientated amino acids [17,19,20], in this article we describe the evaluation of a library comprising 115 FRET-peptide substrates for the characterization of bacterial proteases. Furthermore, the library uniquely contains D-amino-acid-containing peptide substrates, which we used to evaluate D-amino acid substrate specificity and to generate proteolytic fingerprints for a wide range 60 different bacterial species, including Gram-positive, Gram-negative, strictly aerobic and strictly anaerobic species.

Materials and methods

Design of FRET peptide library

We developed a protease substrate library containing 115 short, di-amino-acid peptide substrates, including D-amino-acid-containing substrates (Supplemental Table S1). The library consisted of FRET-based fluorogenic peptide substrates, with each substrate comprising two amino acids where substrates consisted of either (i) two L-amino acids, (ii) a C-terminal L-amino acid and an N-terminal D-amino acid, or (iii) two D-amino acids. L-Amino acids are denoted in uppercase letters (X), D-amino acids in lowercase letters (x). All peptides were flanked with an Ahx-linked fluorescein isothiocyanate (FITC) probe

at the N-terminus and a lysine-coupled Dabcyl (KDbc) quencher at the C-terminus. The FITC-Dabcyl combination is widely used for many FRET-related purposes. A proof of concept for this approach was previously published for the detection of *P. gingivalis* [10,21].

FRET- assay

The 115 FRET-peptides used in this study were purchased from PepScan Presto. (Lelystad, The Netherlands) with a purity > 90%, and the identity of the peptides (substrates) was confirmed using mass spectrometry. All peptides were dissolved in 100% dimethyl sulfoxide (DMSO) to protect the peptides from precipitation in the assay. Proteolytic assays were performed in black, clear-bottom, 96-well plates (Corning, Lowell, USA), as described previously [14]. Briefly, a 50 µl sample (i.e., purified enzyme, bacterial culture supernatant or control biological material) was incubated with 1 µl of substrate (800 µM) at 37 ^{o}C. The final concentration of each peptide in the FRET assay was 16 µM. Fluorescence was then read for 60 min at 2 min intervals using a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Protease activity was defined in fluorescence per minute (F/min), and proteolytic activity with an F/min > 5 was defined as a "positive" result.

Background (non-bacterial) proteolytic controls

The detection of non-specific (non-bacterial) proteolytic activity was determined for several biological materials. Each FRET-substrate was incubated separately with 50 µl of either brain heart infusion medium (BHI) medium (BioTrading, Mijdrecht, The Netherlands), pooled human serum (isotonic sterile Apotheek AZL, cat no 902926, The Netherlands), pooled human urine (n=8) or pooled human non-stimulated saliva (n=7). Urine and saliva were collected from healthy individuals. Verbal informed consent was obtained from all volunteers. Because this study did not involve categorising humans by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, no written informed consent was needed. Samples were collected and used anonymously.

Enzymes

To evaluate the differences between the proteolytic activity of enzymes of bacterial, fungal and eukaryotic origin, a range of five purified proteolytic enzymes was screened using the FRET peptide library. These purified enzymes were the bacterial proteases thermolysin (*Bacillus thermoproteolyticus*), collagenase (*Clostridium histolyticum*), lyso-

staphin (*Staphylococcus staphylolyticus*) and achromopeptidase (*Achromobacter lyticus*) and the fungal protease proteinase K (*Tritirachium album*) (Sigma Aldrich, Zwijndrecht, The Netherlands). Porcine trypsin-EDTA (Lonza, Wijchen, The Netherlands) was used as an example of a eukaryotic protease. All proteases were diluted in phosphate buffered saline (PBS, pH 7,5) and used in the FRET assay at a concentration of 100 µg/ml except for porcine trypsin-EDTA, which was used at a concentration of 25 µg/ml.

Bacterial species

The 60 bacterial species used in this study represent a diverse range of bacterial species associated with human disease. These include both aerobic and anaerobic bacteria, as well as representatives of Gram-positive and Gram-negative bacterial species. The specific species investigated are listed in Supplemental Table S2. All bacteria were grown in BHI medium (Bio-Trading, Mijdrecht, The Netherlands) overnight at 37 ºC to stationary phase and the bacteria were pelleted by centrifugation for 10 min at 10,000 x g. The enzyme containing supernatant was filter sterilised through a 0.22 µm filter (Millipore, Amsterdam, The Netherlands), and samples were used immediately or stored at -20°C until required.

Results

Background (non-bacterial) proteolytic controls

Non bacteria-specific proteolysis was determined using pooled urine, pooled human serum and pooled human non-stimulated whole saliva. Midstream urine from healthy controls showed no proteolytic activity against any of the peptides present in the FRET peptide library (Figure 1A). Pooled serum and saliva showed cleavage of nine and 30 substrates, respectively, including LL-, LD- and some DD-substrates, although for most substrates the cleavage rate was low (F/min 5-20) (Figure 1A). In fact, only the all-L-substrates F-R and R-R were cleaved with a relatively high efficiency by proteases present in pooled serum and saliva (79 and 123 F/min (F-R) and 53 or 45 F/min (R-R), respectively) (Figure 1A).

Proteolytic activity of purified enzymes

To determine differences between the proteolytic activity of enzymes of bacterial, fungal and eukaryotic origin, the FRET peptide library was screened using five different purified enzymes. It was observed that a large percentage (76%) of the exclusively L-amino acid

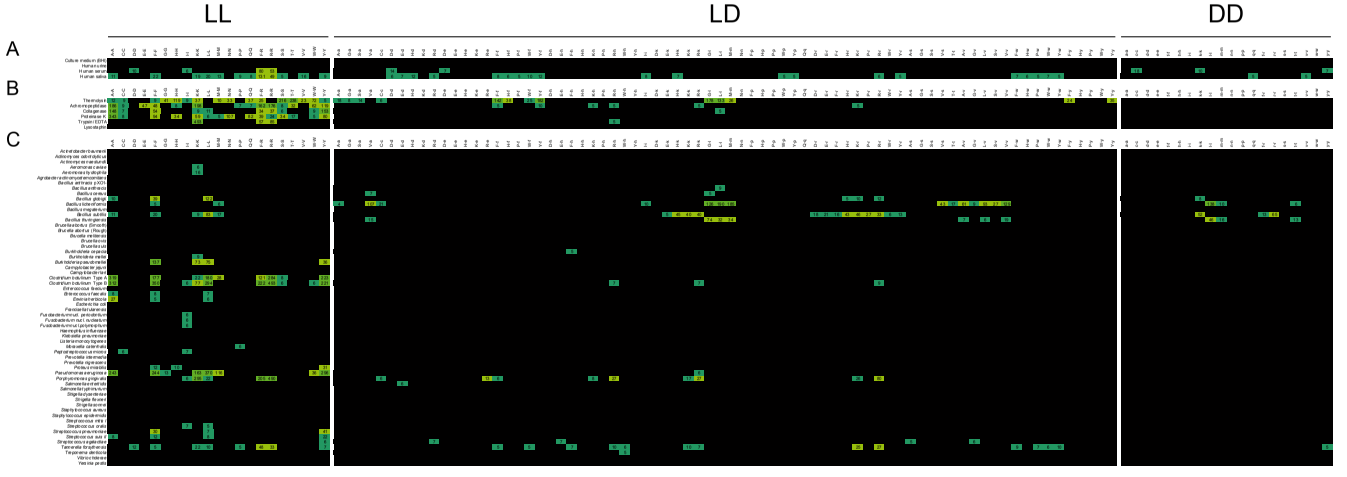

Figure 1. Heat map of the FRET-peptide library screened with background controls, purified enzymes and 60 bacterial culture supernatants. Proteolytic activity of background controls (A), purified enzymes (B) and bacterial culture supernatants (C) on the 115 FRET dipeptide substrates present in the library. Colour scheme: black = lack of activity (F/min < 5); dark green = low activity (F/min 5 to 25), light green = moderate activity (F/min 25 to 125) and neon green = high activity (F/min > 125). L = L-amino acid. D = D-amino acid.

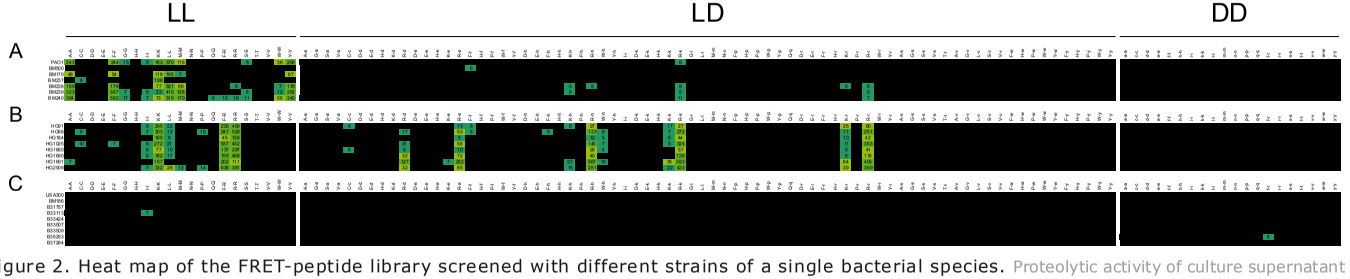

Figure 2. Heat map of the FRET-peptide library screened with different strains of a single bacterial species. Proteolytic activity of culture supernatant from different species of *Porphyromonas gingivalis* (A), *Pseudomonas aeruginosa* (B) and *Staphylococcus aureus* (C). Colour scheme: black = lack of activity (F/min < 5); dark green = low activity (F/min 5 to 25), light green = moderate activity (F/min 25 to125) and neon green = high activity (F/min > 125). L = L-amino acid. D = D-amino acid.

substrates present in the FRET peptide library were efficiently cleaved by thermolysin (Figure 1B). In fact, only substrates D-D, E-E, P-P, L-L and R-R were not cleaved by this enzyme. Of the D-amino-acid-containing substrates, 18% were cleaved by thermolysin, with a preference for the hydrophobic amino acids alanine, phenylalanine, leucine, methionine and tyrosine (Figure 1B).

Similar to thermolysin, achromopeptidase cleaved a large percentage (67%) of the exclusively L-amino acid substrates present in the library, with the uncleaved L-amino acid substrates being D-D, G-G, I-I, L-L, M-M, N-N and V-V. As with thermolysin, achromopeptidase was also able to cleave some (9%) of the D-amino-acid-containing substrates, although the cleavage efficiency (F/min) of these D-amino acid substrates was lower compared with thermolysin. The D-amino acid substrates cleaved by achromopeptidase all consisted of positively charged and/ or aromatic amino acids, with proline being the only exception (Figure 1B).

Of the remaining enzymes tested, the bacterial enzyme collagenase cleaved only the peptides that exclusively consisted of L-amino acids, with no proteolytic activity for D-amino acid substrates. The non-bacterial enzymes proteinase K and trypsin also were unable to cleave D-amino-acid-containing substrates. Collagenase, proteinase K, and trypsin all degraded K-K, R-R and F-R (Figure 1B). Comparison of the total proteolytic activity obtained using the purified enzymes varied widely, with only thermolysin and achromopeptidase being able to cleave substrates in which D-amino acids were present. Furthermore, collagenase and the enzymes from non-bacterial origin, proteinase K and trypsin, exclusively cleaved substrates which consisted of L-amino acids, with each generating their own specific proteolytic "fingerprint" (Figure 1B).

None of the enzymes tested using our FRET peptide library was able to cleave the substrate comprising two aspartic acids, D-D, and no proteolytic activity was observed on any of our FRET-peptide substrates using the bacterial enzyme lysostaphin.

Bacterial proteolytic activity

Screening of bacterial culture supernatants from a wide range of 60 different bacterial species showed that, in general, bacterial proteases exhibit higher proteolytic activity against the all-L-amino-acid-containing substrates compared to substrates containing one or two D-amino acids (Figure 1C). Furthermore, the bacterial culture supernatants tended to show higher and more diverse cleavage activity than the biological materials tested, including bacterial culture broth, pooled human serum, pooled human urine and pooled saliva. However, some of the cleavage activity observed in the pooled saliva

could be attributable to the presence of the normal mouth flora present in the pooled sample.

Of all the bacterial species tested, species of the genera *Bacillus* and *Porphyromonas* were particularly active against many of the peptide substrates present in our FRET peptide library. In particular, culture supernatants of *Bacillus* spp. showed very active proteolytic activity against several of the LL-, LD- and DD-substrates. Furthermore, the D-amino acid substrates containing k-k, l-l and r-r were cleaved by proteases from *Bacillus spp.*, in agreement with previous data [14]. *P. gingivalis* culture supernatant showed a relative broad proteolytic activity on the LD-amino-acid-containing substrates, with 12% of the D-amino acid substrates being cleaved. Furthermore, this proteolytic specificity was relatively conserved among the eight *P. gingivalis* strains examined (Figure 2A).

Analysis of the complete proteolytic profiles of all bacterial supernatants tested revealed novel proteolytic fingerprints between different bacterial species. For example, it was found that the H-H substrate was exclusively cleaved by our clinical isolate of *Proteus mirabilis*, a bacterium involved in urinary tract infections. Interestingly, the proteases of all the other bacterial species tested (Figure 1C) were unable to degrade this H-H substrate. In addition, the G-G substrate was found to be exclusively cleaved by *Pseudomonas aeruginosa* PAO1 (Figure 1C). However, additional experiments revealed that differences existed in the ability of various *P. aeruginosa* strains to cleave this G-G substrate, as well as variation in the proteolytic fingerprints obtained using various *P. aeruginosa* strains. In particular, strains PAO1, BM179, BM238, BM239 and BM240 produced proteases with high cleavage activity on a range of LL- substrates, whereas strains

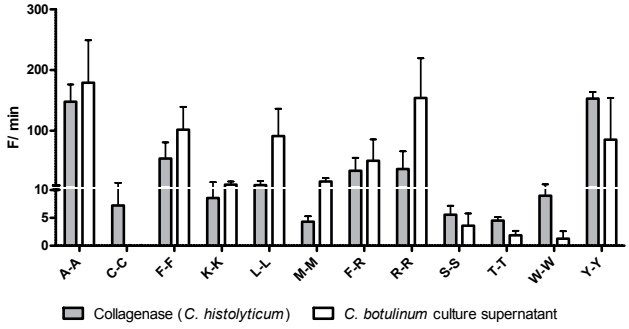

Figure 3. Characterization of the proteolytic activity of *Clostridium histolyticum* derived collagenase and *Clostridium botulinum* culture supernatant on the FRET-peptide library. FRET-peptide substrates cleaved with an efficiency above background (F/min > 5) by the purified enzyme collagenase derived from *C. histolyticum* (grey bars) compared with a culture supernatant obtained from the related bacterium *C. botulinum* type A (white bars). Results are expressed as mean ± standard error of the mean (n=3).

BM500 and BM237 showed very low proteolytic activity against the substrates tested (Figure 2B). This observation highlights the difficulty in attempting to use proteolytic fingerprinting in downstream applications related to, for example, microbial diagnostics of groups of bacterial pathogens.

Cleavage of F-F, K-K, L-L, M-M and Y-Y substrates was observed by *Burkholderia pseudomallei* culture supernatant, although screening of the FRET peptide library with culture supernatants from two additional species of *Burkholderia* revealed that only the K-K substrate was cleaved by supernatants of *B. mallei* and that none of these substrates was cleaved by *B. cepacia*. Therefore, in this initial screening experiment, the substrates F-F, LL, M-M and Y-Y were specific for the proteases secreted by our *B. pseudomallei* strain (Figure 1C). However, as mentioned above, further screening is required using a wide range of *B. pseudomallei* isolates from varied geographical regions.

Culture supernatants of *C. botulinum* (type A and B) showed relatively high proteolytic cleavage activity on 42% of the all-L-amino acid FRET-substrates (Figure 1C), with seven out of the nine substrates being cleaved with an efficiency greater than 20 F/min. Furthermore, no large differences were observed between the proteolytic fingerprints of the two *C. botulinum* types (A and B) examined. The cleavage pattern of *C. botulinum* culture supernatants showed a high degree of similarity with the cleavage pattern obtained using the purified enzyme collagenase, that is derived from the bacterium *C. histolyticum*, with the majority of substrates that were cleaved by *C. histolyticum* collagenase having an efficiency of F/min greater than 5, also being cleaved by culture supernatants of *C. botulinum* (67%) (Figure 3). This finding indicates the presence of a *C. histolyticum*-type collagenase-related protease in the culture supernatants of *C. botulinum.*

In general, the rate of substrate cleavage varied between the bacterial species examined, with *C. botulinum*, *P. gingivalis* and *P. aeruginosa* culture supernatants containing proteases with high proteolytic activity to the peptide substrates present in the FRET peptide library. In contrast, *Brucella* spp., *Yersinia pestis* and *Staphylococcus aureus* culture supernatants were unable to cleave any of the substrates present in the library (Figure 1). This general lack of proteolytic activity by both *S. aureus* and *Y. pestis* on the FRET peptide library was shown not to be restricted to a single strain of these bacterial species, given that additional screening of several different *S. aureus* (Figure 2C) and *Y. pestis* (data not shown) strains also indicated a general lack of proteolytic cleavage against the FRET peptide library.

Discussion

The design and evaluation of peptide substrate screening libraries for the detection of protease activity is not a new phenomenon [17,20,22–24]. However, in general, the screening libraries previously used to characterize proteolytic activity contained substrates comprising five or more amino acids [17,19,20]. This means that each substrate contains multiple potential cleavage sites, making it difficult to determine the exact cleavage site of the substrate without the additional use of mass spectrometry [12]. In contrast, the peptides present in the FRET peptide library designed and evaluated in this article, comprised short di-amino acid substrates that allow simple identification of the proteolytic cleavage site to be determined. Furthermore, the FRET peptide library developed in this article included proteolytic substrates comprising LD- and DD-amino acids in order to evaluate the ability of bacterial proteases to cleave unusual LD- and DD-amino-acid-containing peptides. The composition of this FRET peptide library, together with the extensive range of more than 60 different bacterial species screened, has yielded unique information on (i) the ability of different bacterial species to secrete proteases into their culture environments (ii) the ability of these proteases to cleave novel LD- and DD-amino acid combinations, and (iii) the possible use of proteolytic cleavage profiles (proteolytic "fingerprints") for the future development of proteolytic-based bacterial identification and diagnostics. Although it should be kept in mind that the design of the library is limited, no DL-amino acid combinations are present. In addition, the large FITC and Dabcyl labels could hamper cleavage due to the shortness of the peptides present in the library.

Irrespective of the high numbers of proteases known to be present in serum and saliva [25–27], these specimens showed limited proteolytic activity on our FRET peptide library. However two substrates, R-R and F-R, were efficiently cleaved by both saliva and serum. Cleavage of these substrates by saliva is probably attributable to the presence of cathepsins, enzymes known to degrade a broad range of peptide bonds including arginine (R) and phenylalanine (F) [28], and known to be present in saliva [29]. In human serum, the protease dipeptidyl-peptidase III (DPP3) may be responsible for the cleavage of these substrates [30]. This enzyme prefers the degradation of peptide bonds in which arginine (R) and/ or phenylalanine (F) is present [28]. Overall, proteases in human serum and saliva showed preference for LL-amino acid substrates, with 15% and 58% of the LL-substrates tested being cleaved, respectively. On replacement of a single L-amino acid by a D-amino acid, activity decreased to 5% and 26%, respectively. When substrates consisted of two D-amino acids, saliva showed no proteolytic activity.

With respect to pooled urine, this excellent biological growth medium totally lacked observable proteolytic activity against any of the substrates present in the FRET peptide library. This observation was most probably related to the fact that the pooled urine comprised midstream urine collected from healthy individuals, and also the fact that normal skin commensals (which could contaminate urine), such as *Staphylococcus epidermidis* and *S. aureus*, showed very little proteolytic activity using the substrates available in our FRET peptide library.

Screening the library with commercially available enzymes of both eukaryotic and prokaryotic origin underlined the fact that only bacterial enzymes, although not all bacterial enzymes, have the capacity to cleave D- amino-acid-containing peptides and proteins, with the fungal proteinase K and porcine trypsin both being incapable of cleaving the D-amino-acid-containing substrates present in our FRET-peptide library. Interestingly, of the three bacterial enzymes examined, only collagenase from *C. histolyticum* failed to cleave D-amino-acid-containing substrates. This observation may be related to the different functions of the three different proteases tested. Both bacterial virulence factors (e.g., collagenase) and eukaryotic enzymes are known to target eukaryotic proteins, which predominantly consist of L-orientated amino acids [22,31]. In contrast, the bacterial enzymes involved in housekeeping processes, such as cell wall degradation (e.g., thermolysin and achromopeptidase) [32,33], possess cleavage activity against the D-amino-acid-containing substrates. These D-amino acids are important building blocks of the bacterial cell wall and housekeeping enzymes process D-amino acids bonds for use in cell wall synthesis.

FRET peptide library screening was also performed on a range of 60 different bacterial culture supernatants, where it was observed that bacterial proteases tended to possess relatively high cleavage activity against the LL- amino acid-substrates compared to LD- and DD-containing amino acid substrates. The fact that most of the proteases secreted by bacteria serve as virulence factors, rather than being involved in housekeeping activities probably explains the higher cleavage activity observed for the all-L-amino-acid-containing substrates.

One particular problem associated with the use of phenotypic parameters for microbial identification is that gene expression can vary between bacterial isolates or even within the same bacterial isolate. For example, initial screening of the 60 bacterial culture supernatants revealed a substrate that appeared to be specific for the bacterium *P. aeruginosa* (G-G, glycine-glycine). However, it was shown using supplementary experiments that not all *P. aeruginosa* isolates tested were able to cleave this G-G

substrate. However, repeat screening of three separate culture supernatants of the same *P. aeruginosa* isolate, under identical growth conditions, revealed reproducible cleavage of this G-G substrate (data not shown), indicating that reproducible proteolytic fingerprinting has the potential to be applied in microbial diagnostics.

The use of FRET-peptide screening substrate libraries is not restricted to obtaining microbial fingerprints for possible bacterial diagnosis: it also helps to provide information on potentially novel proteases secreted by bacteria. In this respect, screening results from our FRET peptide library revealed that a substrate consisting of two histidine residues (H-H) was cleaved by the clinical *P. mirabilis* isolate used. There are no *P. mirabilis* specific peptidases described in literature that are known to cleave histidine bonds. This shows the broad applicability of our FRET substrate library in identifying potentially novel bacterial (as well as eukaryotic) proteases. Furthermore, the discovery and characterization of novel proteases could lead to potentially interesting therapeutic targets to fight bacterial infections because bacterial proteases are often indispensable for bacterial growth and virulence [34,35]. By discovering, or developing, novel bacterial protease inhibitors, the pathogenic effects of bacterial virulence factors could possibly be reduced or negated. Protease inhibitors have already proven to be effective in the treatment of various viral infections such as HIV and hepatitis C [34]. In both cases, the clinically approved protease inhibitors function via a competition mechanism [36,37]. The use of FRET-screening libraries will help in the search towards such competitive protease inhibitors.

In conclusion, the authors have developed and evaluated a simple, rapid, and high-throughput methodology to screen for bacterial proteolytic activity. The use and characterization of L- and D-amino acid proteolytic profiles for use with specific bacterial strains within a species, as well as for use with clinical materials and samples from different environmental backgrounds, will help facilitate the more rapid identification of bacterial proteases that possess novel cleavage patterns. Furthermore, the use of such FRET peptide substrate libraries allows insights with respect to the specificity and efficiency of inhibition of novel protease inhibitors. Research into both proteolytic enzyme cleavage patterns, as well as the characteristics of novel proteolytic cleavage inhibitors, will help to identify new proteases for exploitation in future medical and biotechnological applications.

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Supplemental data

Table S1. FRET-peptide substrate library

	Sequence		Sequence		Sequence
PFU-001	FITC-Ahx-A-a-KDbc	PFU-049	FITC-Ahx-H-r-KDbc	PFV-001	FITC-Ahx-c-c-KDbc
PFU-002	FITC-Ahx-G-a-KDbc	PFU-050	FITC-Ahx-K-r-KDbc	PFV-002	FITC-Ahx-d-d-KDbc
PFU-003	FITC-Ahx-S-a-KDbc	PFU-051	FITC-Ahx-P-r-KDbc	PFV-003	FITC-Ahx-e-e-KDbc
PFU-004	FITC-Ahx-V-a-KDbc	PFU-052	FITC-Ahx-R-r-KDbc	PFV-004	FITC-Ahx-f-f-KDbc
PFU-005	FITC-Ahx-C-c-KDbc	PFU-053	FITC-Ahx-W-r-KDbc	PFV-005	FITC-Ahx-h-h-KDbc
PFU-006	FITC-Ahx-D-d-KDbc	PFU-054	FITC-Ahx-Y-r-KDbc	PFV-006	FITC-Ahx-i-i-KDbc
PFU-007	FITC-Ahx-E-d-KDbc	PFU-055	FITC-Ahx-A-s-KDbc	PFV-007	FITC-Ahx-k-k-KDbc
PFU-008	FITC-Ahx-H-d-KDbc	PFU-056	FITC-Ahx-G-s-KDbc	PFV-008	FITC-Ahx-l-l-KDbc
PFU-009	FITC-Ahx-K-d-KDbc	PFU-057	FITC-Ahx-S-s-KDbc	PFV-009	FITC-Ahx-m-m-KDbc
PFU-010	FITC-Ahx-R-d-KDbc	PFU-058	FITC-Ahx-V-s-KDbc	PFV-010	FITC-Ahx-n-n-KDbc
PFU-011	FITC-Ahx-D-e-KDbc	PFU-059	FITC-Ahx-T-t-KDbc	PFV-011	FITC-Ahx-p-p-KDbc
PFU-012	FITC-Ahx-E-e-KDbc	PFU-060	FITC-Ahx-A-v-KDbc	PFV-012	FITC-Ahx-q-q-KDbc
PFU-013	FITC-Ahx-H-e-KDbc	PFU-061	FITC-Ahx-G-v-KDbc	PFV-013	FITC-Ahx-f-r-KDbc
PFU-014	FITC-Ahx-K-e-KDbc	PFU-062	FITC-Ahx-L-v-KDbc	PFV-014	FITC-Ahx-r-r-KDbc
PFU-015	FITC-Ahx-R-e-KDbc	PFU-063	FITC-Ahx-S-v-KDbc	PFV-015	FITC-Ahx-s-s-KDbc
PFU-016	FITC-Ahx-F-f-KDbc	PFU-064	FITC-Ahx-V-v-KDbc	PFV-016	FITC-Ahx-t-t-KDbc
PFU-017	FITC-Ahx-H-f-KDbc	PFU-065	FITC-Ahx-F-w-KDbc	PFV-017	FITC-Ahx-v-v-KDbc
PFU-018	FITC-Ahx-P-f-KDbc	PFU-066	FITC-Ahx-H-w-KDbc	PFV-018	FITC-Ahx-w-w-KDbc
PFU-019	FITC-Ahx-W-f-KDbc	PFU-067	FITC-Ahx-P-w-KDbc	PFV-019	FITC-Ahx-y-y-KDbc
PFU-020	FITC-Ahx-Y-f-KDbc	PFU-068	FITC-Ahx-W-w-KDbc		
PFU-021	FITC-Ahx-D-h-KDbc	PFU-069	FITC-Ahx-Y-w-KDbc		
PFU-022	FITC-Ahx-E-h-KDbc	PFU-070	FITC-Ahx-F-y-KDbc		
PFU-023	FITC-Ahx-F-h-KDbc	PFU-071	FITC-Ahx-H-y-KDbc		
PFU-024	FITC-Ahx-H-h-KDbc	PFU-072	FITC-Ahx-P-y-KDbc		
PFU-025	FITC-Ahx-K-h-KDbc	PFU-073	FITC-Ahx-W-y-KDbc		
PFU-026	FITC-Ahx-P-h-KDbc	PFU-074	FITC-Ahx-Y-y-KDbc		
PFU-027	FITC-Ahx-R-h-KDbc	PFU-075	FITC-Ahx-A-A-KDbc		
PFU-028	FITC-Ahx-W-h-KDbc	PFU-076	FITC-Ahx-C-C-KDbc		
PFU-029	FITC-Ahx-Y-h-KDbc	PFU-077	FITC-Ahx-D-D-KDbc		
PFU-030	FITC-Ahx-I-i-KDbc	PFU-078	FITC-Ahx-E-E-KDbc		
PFU-031	FITC-Ahx-D-k-KDbc	PFU-079	FITC-Ahx-F-F-KDbc		
PFU-032	FITC-Ahx-E-k-KDbc	PFU-080	FITC-Ahx-G-G-KDbc		
PFU-033	FITC-Ahx-H-k-KDbc	PFU-081	FITC-Ahx-H-H-KDbc		
PFU-034	FITC-Ahx-K-k-KDbc	PFU-082	FITC-Ahx-I-I-KDbc		
PFU-035	FITC-Ahx-R-k-KDbc	PFU-083	FITC-Ahx-K-K-KDbc		
PFU-036	FITC-Ahx-G-l-KDbc	PFU-084	FITC-Ahx-L-L-KDbc		
PFU-037	FITC-Ahx-L-l-KDbc	PFU-085	FITC-Ahx-M-M-KDbc		
PFU-038	FITC-Ahx-M-m-KDbc	PFU-086	FITC-Ahx-N-N-KDbc		
PFU-039	FITC-Ahx-N-n-KDbc	PFU-087	FITC-Ahx-P-P-KDbc		
PFU-040	FITC-Ahx-F-p-KDbc	PFU-088	FITC-Ahx-Q-Q-KDbc		
PFU-041	FITC-Ahx-H-p-KDbc	PFU-089	FITC-Ahx-F-R-KDbc		
PFU-042	FITC-Ahx-P-p-KDbc	PFU-090	FITC-Ahx-R-R-KDbc		
PFU-043	FITC-Ahx-W-p-KDbc	PFU-091	FITC-Ahx-S-S-KDbc		
PFU-044	FITC-Ahx-Y-p-KDbc	PFU-092	FITC-Ahx-T-T-KDbc		
PFU-045	FITC-Ahx-Q-q-KDbc	PFU-093	FITC-Ahx-V-V-KDbc		
PFU-046	FITC-Ahx-D-r-KDbc	PFU-094	FITC-Ahx-W-W-KDbc		
PFU-047	FITC-Ahx-E-r-KDbc	PFU-095	FITC-Ahx-Y-Y-KDbc		
PFU-048	FITC-Ahx-F-r-KDbc	PFU-096	FITC-Ahx-a-a-KDbc		

Table S2. Bacterial strains used in this study [a]

Strain	
Acinetobacter baumanii	Clinical isolate
Actinomyces naeslundii	ATCC 12104
Actinomyces odontolyticus	HG472
Aeromonas caviae	Clinical isolate
Aeromonas hydrophila	Clinical isolate
Aggregatibacter actinomycetemcomitans	NCTC 9710
Bacillus anthracis	ATCC 14578
Bacillus anthracis	ATCC 4229, pXO1^{-}
Bacillus cereus	ATCC 14579
Bacillus globigii	BM013, TNO collection
Bacillus licheniformis	ATCC 11560
Bacillus megaterium	ATCC 15374
Bacillus subtilis	ATCC 6051
Bacillus thuringiensis	Var. kurstaki aizawai
Brucella abortus	Smooth, B19
Brucella abortus	Rough, 45/20
Brucella melitensis	ATCC 23456, 16M
Brucella ovis	63/290
Brucella suis	ATCC 23444, 1330
Burkholderia cepacia	ATCC 25416
Burkholderia mallei	ATCC 10399
Burkholderia pseudomallei	ATCC 23343
Campylobacter jejuni	ATCC 33560
Campylobacter lari	ATCC 35221
Clostridium botulinum	NCTC 2916, type A
Clostridium botulinum	NCTC 7273, type B
Enterococcus faecium	Clinical isolate, VanA
Enterococcus faecium	Clinical isolate
Enterococcus faecalis	Clinical isolate
Erwinia herbicola	ATCC 33243
Escherichia coli	ATCC 25922
Francisella tularensis	
Fusobacterium nucleatum subsp. periodontium	ATCC 33693
Fusobacterium nucleatum subsp. nucleatum	ATCC 25586
Fusobacterium nucleatum subsp. polymorphum	ATCC 10953
Haemophilus influenzae	Clinical isolate
Klebsiella pneumoniae	Clinical isolate
Listeria monocytogenes	EGDe, BAA-679
Moraxella caterrhalis	Clinical isolate
Peptostreptococcus micros	ATCC 33270
Prevotella intermedia	ATCC 25611
Prevotella nigrescens	NCTC 9336

Proteus mirabilis	Clinical isolate
Pseudomonas aeruginosa	PAO1, ATCC 15692
Pseudomonas aeruginosa	BM500, ATCC 27853, TNO collection
Pseudomonas aeruginosa	BM179, Clinical isolate, TNO collection
Pseudomonas aeruginosa	BM237, Clinical isolate, TNO collection
Pseudomonas aeruginosa	BM238, Clinical isolate, TNO collection
Pseudomonas aeruginosa	BM239, Clinical isolate, TNO collection
Pseudomonas aeruginosa	BM240, Clinical isolate, TNO collection
Porphyromonas gingivalis	HG66, W83
Porphyromonas gingivalis	HG91, 381
Porphyromonas gingivalis	HG184, X-2
Porphyromonas gingivalis	HG1025, A7A1-28
Porphyromonas gingivalis	HG1660, ATCC 49417
Porphyromonas gingivalis	HG1690
Porphyromonas gingivalis	HG1691
Porphyromonas gingivalis	HG2909, 34-4
Shigella dysenteriae	Clinical isolate
Shigella flexneri	Clinical isolate
Shigella sonnei	Clinical isolate
Salmonella enteritidis	Clinical isolate
Salmonella typhimurium	ATCC 25416
Staphylococcus aureus	USA300
Staphylococcus aureus	BM186, Clinical isolate, TNO collection
Staphylococcus aureus	B31757, Clinical isolate
Staphylococcus aureus	B33113, Clinical isolate
Staphylococcus aureus	B33424, Clinical isolate
Staphylococcus aureus	B33507, Clinical isolate
Staphylococcus aureus	B33509, Clinical isolate
Staphylococcus aureus	B35253, Clinical isolate
Staphylococcus aureus	B37264, Clinical isolate
Streptococcus mitis I	SK 95
Streptococcus oralis	SK 1477
Streptococcus pneumoniae	Clinical isolate
Streptococcus suis II	Clinical isolate
Streptococcus agalactiae	Clinical isolate
Tannerella forsythia	ATCC 43037
Treponema denticola	ATCC 35405
Vibrio cholerae	ATCC 14035
Yersinia pestis	NCTC 10329

[a] All bacteria were grown in Brain Heart Infusion (BHI) broth at 37 °C. *Porphyromonas gingivalis, Actinomyces odontolyticus, Aggregetobacter actinomycetemcomitans, Prevotella intermedia, Prevotella nigrescens, Tannerella forsythensis, Peptostreptococcus micros, Fusobacterium nucleatum periodontium, Fusobacterium nucleatum nucleatum, Fusobacterium nucleatum polymorphum, Streptococcus mitis I, Streptococcus oralis, Actinomyces naeslundii, Campylobacter jejuni, Campylobacter lari* and *Clostridium botulinum* (type A and B) were grown under anaerobic conditions. *P. gingivalis* was grown in BHI supplemented with 1 µg/ml hemin and 0.5 µg/ml menadione (Sigma, Zwijndrecht, The Netherlands). *Brucella abortus, Brucella melitensis, Brucella ovis, Brucella suis*, and *Haemophilus influenzae* were grown in presence of 5% CO_2.

Chapter 3

Highly specific protease-based approach for detection of *Porphyromonas gingivalis* in diagnosis of periodontitis

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Abstract

Porphyromonas gingivalis is associated with the development of periodontitis. Here we describe the development of a highly specific protease-based diagnostic method for the detection of *P. gingivalis* in gingival crevicular fluid. Screening of a proteolytic peptide substrate library, including fluorogenic dipeptides that contain D-amino acids, led to the discovery of five *P. gingivalis* specific substrates. Due to the presence of lysine and arginine residues in these substrates, it was hypothesised that the cleavage was mediated by the gingipains, a group of *P. gingivalis*-specific proteases. This hypothesis was confirmed by the observation that *P. gingivalis* gingipain knockout strains demonstrated clearly impaired substrate cleavage efficacy. Further, proteolytic activity on the substrates was increased by the addition of the gingipain stimulators dithiothreitol and L-cysteine and decreased by the inhibitors leupeptin and *N*-ethylmaleimide. Screening of saliva and gingival crevicular fluid of periodontitis patients and healthy controls showed the potential of the substrates to diagnose the presence of *P. gingivalis* proteases. By using paper points, a sensitivity of approximately 10^5 CFU/ml was achieved. *P. gingivalis*-reactive substrates fully composed of L-amino acids and Bz-L-Arg-NHPhNO2 showed a relatively low specificity (44 to 85%). However, the five *P. gingivalis*-specific substrates that each contained a single D-amino acid showed high specificity (96 to 100%). This observation underlines the importance of the presence of D-amino acids in substrates used for the detection of bacterial proteases. We envisage that these substrates may improve the specificity of the current enzyme-based diagnosis of periodontitis associated with *P. gingivalis*.

Introduction

Periodontitis is an inflammation of the periodontium, the tissues that surround and support the teeth. Severe periodontitis affects at least 10% of the general population and involves progressive loss of the alveolar bone around the teeth [17,18,29]. If left untreated, periodontitis can lead to loosening and subsequent loss of teeth [45]. Periodontitis is induced by microorganisms that adhere to and grow at the gingival margin, along with an overly aggressive immune response against these microorganisms [9]. The microorganisms implicated in periodontitis include *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tannerella forsythia* and *Treponema denticola* [11,13,26,31]. Periodontal pathogens, in particular, *T. denticola*, *T. forsythia* and *P. gingivalis,* secrete protease virulence factors that allow these bacteria to invade the host's tissues [13,16,21,25,37]. By liberating amino acids from host proteins, secreted proteases are actively involved in the (anaerobic) metabolism of these bacteria. In addition to being a major cause of tooth loss, periodontal disease has also been associated with several systemic diseases. Animal- and population-based studies have demonstrated associations between periodontal disease and diabetes, cardiovascular disease, rheumatoid arthritis, stroke, and adverse pregnancy outcomes [4,8,38]. Although the mechanisms that cause these associations are far from being completely understood, more reliable detection of early stage periodontal disease could have widespread health benefits, even beyond the prevention of tooth loss.

Despite today's widespread occurrence of periodontal disease, currently available diagnostic tests are limited in their sensitivity and specificity [46]. The best available diagnostic aid is measurement of the depth of the tooth pocket, but this provides a retrospective analysis only and mostly when tooth attachment is already lost. Moreover, this is a mere effect measurement and this does not help to identify causative agents. Culture-based, nucleic acid-based and antibody-based diagnostic methods are available to help identify the periodontal pathogens involved, which is important to determine suitable therapeutic procedures invoking the highest chance of therapeutic success [6,14,19]. Still, cultivation or using nucleic acid-based and immunochemical tests to identify periodontal pathogens can be very laborious and time-consuming. So far, direct detection and identification of periodontal pathogens *in situ* has proven difficult.

Therefore, the goal of this study was to develop a rapid and simple diagnostic technology that would enable the dental practitioner to perform a "chair-side" test to identify the presence of periodontal pathogens with a focus on *P. gingivalis.* The application of such a test based on the enzymatic diagnosis of periodontal pathogens has

already been described by Loesche and co-workers [27]. Using the benzoyl-DL-arginine-naphthylamide (BANA) substrate it is possible to detect *T. denticola*, *T. forsythia* and *P. gingivalis* by their proteolytic activity in plaque samples [28]. To start suitable treatment of periodontitis, it is important to know which pathogen is involved. Nevertheless using the BANA substrate, it is not possible to distinguish among these three bacterial species and exclusively detect *P. gingivalis*.

We showed in a previous study that the introduction of a D-amino acid residue in a peptide substrate enhanced the specificity of a test that was geared toward the detection of proteolytic activity specifically associated with a certain bacterial species [20]. The all-L-amino acid peptide fluorescein isothiocyanate (FITC)-Leu-Leu-Dabcyl (KDbc) was cleaved by a wide range of bacterial proteases. However, when one of the leucins within the substrate was substituted by its D-enantiomer, the peptide was exclusively cleaved by *Bacillus* ssp. and not by any of the other species tested [20]. Further, in contrast to the all-L-amino acid parental substrate, the variant was not degraded by enzymes present in serum and saliva. Essentially, the introduction of D-amino acids in a peptide aids to design substrates that are bacterial species-specific due to their presence as a component of the bacterial cell wall [43]. In contrast, D-amino acids are, with only a few exceptions, not metabolized in eukaryotic cells [33]. In our view, this approach has opened a novel, attractive avenue to develop more bacterial species-specific substrates that can be applied in the diagnosis of infectious agents in complex matrices. Based on the study on D-amino-acid-containing peptides for the detection of *Bacillus* spp., we developed a substrate library containing fluorogenic dipeptides that contain no, one, or two D-amino acids. We used this approach to shotgun screen for substrates that react specifically with *P. gingivalis* proteases.

Materials and methods

Bacteria

The bacterial strains used in this study are listed in Table 1. Bacteria were grown in 15 ml brain heart infusion (BHI) medium (BioTrading, Mijdrecht, The Netherlands) under anaerobic conditions at 37 °C. All *P. gingivalis* cultures were supplemented with 1 µg/ml hemin and 0.5 µg/ml menadione (Sigma, Zwijndrecht, The Netherlands). After 72 h of culturing, the bacteria were pelleted by centrifugation for 10 min at 10,000 x g. The supernatant, containing secreted enzymes, was sterilised by filtration through

Table 1. Bacterial strains used in this study

Strain			Reference
Porphyromonas gingivalis	W50		
Porphyromonas gingivalis	HG91	Non-encapsulated K-	[23,24]
Porphyromonas gingivalis	W83	Capsular serotype K1	[23,24]
Porphyromonas gingivalis	HG184	Capsular serotype K2	[23,24]
Porphyromonas gingivalis	A7A1-28	Capsular serotype K3	[23,24]
Porphyromonas gingivalis	ATCC 49417	Capsular serotype K4	[23,24]
Porphyromonas gingivalis	HG1690	Capsular serotype K5	[23,24]
Porphyromonas gingivalis	HG1691	Capsular serotype K6	[23,24]
Porphyromonas gingivalis	34-4	Capsular serotype K7	[3]
Porphyromonas gingivalis	K1A	Δ*Kgp*	[1]
Porphyromonas gingivalis	KDP133	Δ*RgpA*/Δ*RgpB*	[41]
Porphyromonas gingivalis	KDP136	Δ*Kgp*/Δ*RgpA*/Δ*RgpB*	[41]
Actinomyces naeslundii		ATCC 12104	
Actinomyces odontolyticus		HG472	
Aggregatibacter actinomycetemcomitans		NCTC 9710	
Fusobacterium nucleatum subsp. *periodontium*		ATCC 33693	
Fusobacterium nucleatum subsp. *nucleatum*		ATCC 25586	
Fusobacterium nucleatum subsp. *polymorphum*		ATCC 10953	
Peptostreptococcus micros		ATCC 33270	
Prevotella intermedia		ATCC 25611	
Prevotella nigrescens		NCTC 9336	
Streptococcus mitis I		SK 95	
Streptococcus oralis		SK1477	
Tannerella forsythia		ATCC 43037	
Treponema denticola		ATCC 35405	

a 0.22 µm filter (Millipore, Amsterdam, The Netherlands). Crude samples were used immediately or stored at -20 °C for later use.

FRET- substrates

The 115 novel fluorogenic substrates used were purchased at PepScan Presto (Lelystad, The Netherlands) and were > 90% pure [20,36]. The identity of the substrates was confirmed by mass spectrometry. The *P. gingivalis* specific substrates were denoted as "BikKams" (Table 2). Assays were performed in black clear bottom 96-well plates (Corning, Massachusetts, USA). Proteolytic activity was determined by the addition of 1 µl of substrate (800 µM) to 50 µl of filtered culture supernatant or whole culture for the sensitivity assay. Culture broth was used as a negative control. Plates were read for 60 min at 37 °C with 2 min intervals on a fluorescence microplate reader (FLUOstar Galaxy,

Table 2. Sequences of FRET- library peptides cleaved by *P. gingivalis* culture supernatant

	Sequence
BikKam9	FITC-Arg-D-Asp-KDbc
BikKam10	FITC-Arg-D-Glu-KDbc
BikKam11	FITC-Arg-D-His-KDbc
BikKam12	FITC-Arg-D-Lys-KDbc
BikKam13	FITC-Arg-D-Arg-KDbc
BikKam14	FITC-Lys-Lys-KDbc
BikKam15	FITC-Phe-Arg-KDbc
BikKam16	FITC-Arg-Arg-KDbc

BMG Laboratories, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Relative fluorescence (RF) values were obtained after correction against the culture broth control. The protease activity was defined in RF per minute (RF/min). An RF/min value higher than 5 was considered positive.

Cleavage characteristics of *P. gingivalis* substrates

P. gingivalis strain W50 culture supernatant was prepared as described above. Culture supernatant was incubated with 16 µM of each substrate in the presence of various concentrations of five different chemicals which are known to influence the cleavage activity of gingipains [5,7]. Proteolytic activity was measured as described previously. The compounds used were leupeptin, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), L-cysteine and glycyl-glycine. All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands).

Sensitivity testing of *P. gingivalis* substrates

P. gingivalis W50 was cultured in BHI medium supplemented with 1 µg/ml hemin and 0.5 µg/ml menadione for 72 h under anaerobic conditions at 37 °C. The number of bacteria was determined by plating 10-fold serial dilutions on trypticase soy agar (TSA) plates (BioTrading, Mijdrecht, The Netherlands). Plates were incubated at 37 ºC under anaerobic conditions and bacteria were enumerated after 3 days of incubation. The culture was serially diluted in culture broth (10^9, 10^8, 10^7, 10^6 and 10^5 CFU/ml), and 50 µl of each dilution was used to test the sensitivities of the substrates. Cleavage of the substrates was catalyzed by the addition of 5 µl L-cysteine (50 mM) to each enzyme reaction mixture.

To mimic clinical samples, paper points were spiked with various concentrations of *P. gingivalis* W50. For this purpose, a *P. gingivalis* culture was serially diluted as described above. Paper points were incubated in the diluted culture until saturated and placed into

reduced transport fluid (RTF)- containing vials [44]. Four paper points per dilution were used. After thorough vortexing, 50 µl of suspension was incubated with 1 µl of substrate (800 µM) and 5 µl of L-cysteine (50 mM).

Proteolytic activity was measured as described previously. RF values were obtained after correction against the culture broth or RTF control. The protease activity was defined in RF/min. An RF/min value higher than 5 was considered positive.

Analysis of periodontitis patient paper points

To study the clinical applicability of the substrates to diagnose *P. gingivalis* infections *in situ*, gingival crevicular fluid from 72 patients suffering from periodontitis, sampled using paper points, was utilized. The study was approved by the Institutional Ethical Board of the Academic Hospital Vrije Universiteit at Amsterdam and informed consent was obtained from all donors. Subgingival samples were taken from four different periodontal pockets per patient using paper points. After sampling, the paper points were transferred into RTF-containing vials. After thorough vortexing, 1 µl of substrate (800 µM) and 5 µl of L-cysteine (50 mM) were added to 50 µl of suspension. Plates were read for 90 min at 37 ºC with 2 min intervals on a fluorescence microplate reader as described previously. RF values were obtained after correction against the RTF control. The protease activity was defined RF/min.

Additionally, the RTF, containing dental plaque bacteria, was analysed using Bz-L-Arg-NHPhNO_2 (L-BApNA; PeptaNova, Sandhausen, Germany), a commercially available proteolytic substrate which is commonly used for the diagnosis of periodontal pathogens. For this purpose, 50 µl of suspension was incubated with 4 mM L-BApNA and 5 mM L-cysteine. Plates were incubated at 37 ºC for 60 min and optical density at 405 nm (OD_{405}) was measured using a microplate reader (BioRad Laboratories, Veenendaal, The Netherlands). Paper points with an RF/min value of > 5 or an OD_{405} of > 1.2 were considered positive. Simultaneously, serving as the gold standard, gingival crevicular fluid eluted from the paper points from the same pockets was analysed for the presence of a number of typical periodontal pathogens, including *A. actinomycetemcomitans, P. intermedia, T. forsythia, Peptostreptococcus micros, Fusobacterium nucleatum* and *P. gingivalis,* using standard microbiological culture Sensitivity is defined as the percentage of samples that are BikKam or L-BApNA positive as well as *P. gingivalis* culture positive. Specificity is defined as the percentage of samples which are both BikKam or L-BApNA negative as well as *P. gingivalis* culture negative (< 10 CFU/ml).

Results

Design of a FRET-substrate library

We developed a shotgun substrate library based on our study on D-amino-acid-containing peptides for the detection of *Bacillus* spp. [20]. The library consisted of fluorogenic substrate peptides, each comprising two amino acids where (i) both amino acids were L-amino acids, (ii) the C-terminally located amino acid was a D-amino acid, and (iii) both amino acids were D-amino acids. All peptides were flanked with an amino hexanoic acid (Ahx) -linked fluorescein isothiocyanate (FITC) at the N-terminus and a lysine-coupled Dabcyl (KDbc) quencher at the C-terminus. The library consists of 115 peptides in total (Table S1).

To test the library concept, culture supernatant of *P. gingivalis* was incubated with all FRET-peptides present within the library. Of the 115 peptides, eight peptides were cleaved by the *P. gingivalis* culture supernatant. Strikingly, all *P. gingivalis*-positive substrates contained an arginine or lysine residue (Table 2).

Specificity of the *P. gingivalis* substrates

To examine the specificity of the *P. gingivalis*-positive substrates, the peptides were incubated with culture supernatants from eight different *P. gingivalis* strains and 12 other oral pathogens (Table 3). All eight substrates were cleaved by all of the *P. gingivalis* strains tested, except for BikKam9, which was not cleaved by the culture supernatant of *P. gingivalis* K2. Further, cleavage of the substrates varied in efficiency; BikKam9 and BikKam10 showed the lowest cleavage activity, whereas BikKam14 to BikKam16 showed the highest cleavage activity (Table 3). It was found that the majority of the substrates cleaved by *P. gingivalis* enzymes were not degraded by any of the other oral pathogens tested, the only exception being BikKam15, which was cleaved by proteases present in the culture supernatant of *T. forsythia* (Table 3).

To further evaluate the possibility of the use of these novel substrates in the diagnosis of periodontitis, saliva from seven healthy volunteers was incubated with the eight *P. gingivalis* substrates. As expected, the substrates which consisted of exclusively L-amino acids (BikKam14 to BikKam16) were cleaved by saliva, whereas no cleavage activity was observed when the D-amino-acid-containing substrates were used (Table 3).

Table 3. Proteolytic activities of bacterial culture supernatants against *P. gingivalis* substrates [a]

	BikKam9	BikKam10	BikKam11	BikKam12	BikKam13	BikKam14	BikKam15	BikKam16
P. gingivalis W50	+++	+++	+++	+++	+++	+++	+++	+++
P. gingivalis K-	+	+	++	++	++	+++	+++	+++
P. gingivalis K1	+	++	+++	+++	+++	+++	+++	+++
P. gingivalis K2	-	+	+	++	++	+++	++	+++
P. gingivalis K3	++	++	+++	+++	+++	+++	+++	+++
P. gingivalis K4	+	+	++	++	++	++	+++	+++
P. gingivalis K5	++	++	++	+++	+++	+++	+++	+++
P. gingivalis K6	+++	+++	+++	+++	+++	+++	+++	++
P. gingivalis K7	++	++	+++	+++	+++	+++	+++	+++
A. naeslundii	-	-	-	-	-	-	-	-
A. odontolyticus	-	-	-	-	-	-	-	-
A. actinomycetemcomitans	-	-	-	-	-	-	-	-
F. nucl. subsp. *periodontium*	-	-	-	-	-	-	-	-
F. nucl. subsp. *nucleatum*	-	-	-	-	-	-	-	-
F. nucl. subsp. *polymorphum*	-	-	-	-	-	-	-	-
Peptostreptococcus micros	-	-	-	-	-	-	-	-
Prevotella intermedia	-	-	-	-	-	-	-	-
Prevotella nigrescens	-	-	-	-	-	-	-	-
Streptococcus mitis I	-	-	-	-	-	-	-	-
Streptococcus oralis	-	-	-	-	-	-	-	-
Tannerella forsythia	-	-	-	-	-	-	+	-
Treponema denticola	-	-	-	-	-	-	-	-
Saliva	-	-	-	-	-	+	+++	++

[a] Enzyme activity is defined in RF/min values as follows: < 5 (-), no activity; 5 to 24 (+), low activity; 25-124 (++), moderate activity; > 125 (+++), high activity.

Table 4. Effect of protease inhibitors and stimulators on the cleavage of the FRET-substrates by *P. gingivalis* W50 [a]

	BikKam9	BikKam10	BikKam11	BikKam12	BikKam13	BikKam14	BikKam15	BikKam16
Compound				**Activity (%)**				
None	100	100	100	100	100	100	100	100
Leupeptin (μM)								
25	54	48	28	28	29	102	25	24
50	50	42	26	21	24	101	19	15
100	48	42	23	20	21	97	14	11
NEM (mM)								
6.75	39	27	25	16	14	13	7	4
12.5	30	19	19	11	10	11	4	2
25	23	13	14	8	8	8	3	2
DTT (mM)								
25	213	165	272	267	235	1071	364	562
50	275	273	500	564	562	1570	683	1164
100	399	340	709	927	886	1962	1197	1722
L-Cysteine								
25	213	292	570	701	811	2728	977	1920
50	250	334	672	842	920	2494	952	1981
100	274	403	849	899	969	2855	1027	2185
Glycyl-glycine (mM)								
25	98	96	93	99	99	99	96	103
50	97	99	91	99	96	95	89	104
100	96	97	93	94	95	100	92	100

[a] Inhibitors: leupeptin, NEM. Stimulators: DTT, L-cysteine, and glycyl-glycine.

Mapping of the proteolytic characteristics of the *P. gingivalis* FRET-substrates

Of the substrates present in the library, only the peptides which contained arginine or lysine residues were cleaved by the culture supernatant of *P. gingivalis.* From this observation, we hypothesised that the enzymes responsible for the cleavage of the substrates could be the *P. gingivalis* specific Arg-gingipain and Lys-gingipain peptidases. These gingipains are members of the cysteine peptidase family. Therefore, we verified in addition whether (i) the addition of L-cysteine and DTT could stimulate cleavage, and (ii) inhibitors such as NEM and leupeptin could inhibit proteolytic activity.

The addition of DTT and L-cysteine resulted in the increased degradation of all substrates (Table 4). Especially the presence of L-cysteine led to a significant increase in activity of BikKam14 and BikKam16 cleavage (> 10- to 28-fold compared to the control). In line, decreased proteolytic activity was observed when the reaction was performed in the presence of NEM or leupeptin. The cleavage activity of BikKam14 in the presence of leupeptin was unaffected (Table 4). No significant increase in substrate cleavage was observed when the gingipain stimulator glycyl-glycine was added to the reaction mixture (Table 4).

Proteolytic activity of the *P. gingivalis* gingipain knockout strains

To further explore the role of gingipains in the cleavage of the artificial substrates, the activity of culture supernatants of *P. gingivalis* strains which lack the Arg-gingipains (ΔRgp), Lys-gingipain (ΔKgp), or both (ΔRgp/ ΔKgp) was examined. In both ΔRgp and ΔRgp/ ΔKgp supernatants, cleavage of the arginine containing substrates was absent (Figure 1A to E, G and H) The substrates which consists of only lysine residues, BikKam14, did show cleavage by ΔRgp though it was with a much lower efficiency compared to the wild-type strain (Figure 1F). As expected, there was no cleavage of BikKam14 by ΔKgp or ΔRgp/ΔKgp (Figure 1F). Surprisingly, besides BikKam14 also decreased efficiency of cleavage by ΔKgp was observed on the other substrates (Figure 1A to E, G and H).

Sensitivity of the *P. gingivalis* substrates

To study the sensitivity of the *P. gingivalis* specific protease test, we analysed serial dilutions of *P. gingivalis* culture using all eight substrates. BikKam9 and BikKam10 showed the lowest sensitivity with a limit of detection of 10^8 CFU/ml (Figure 2A). The

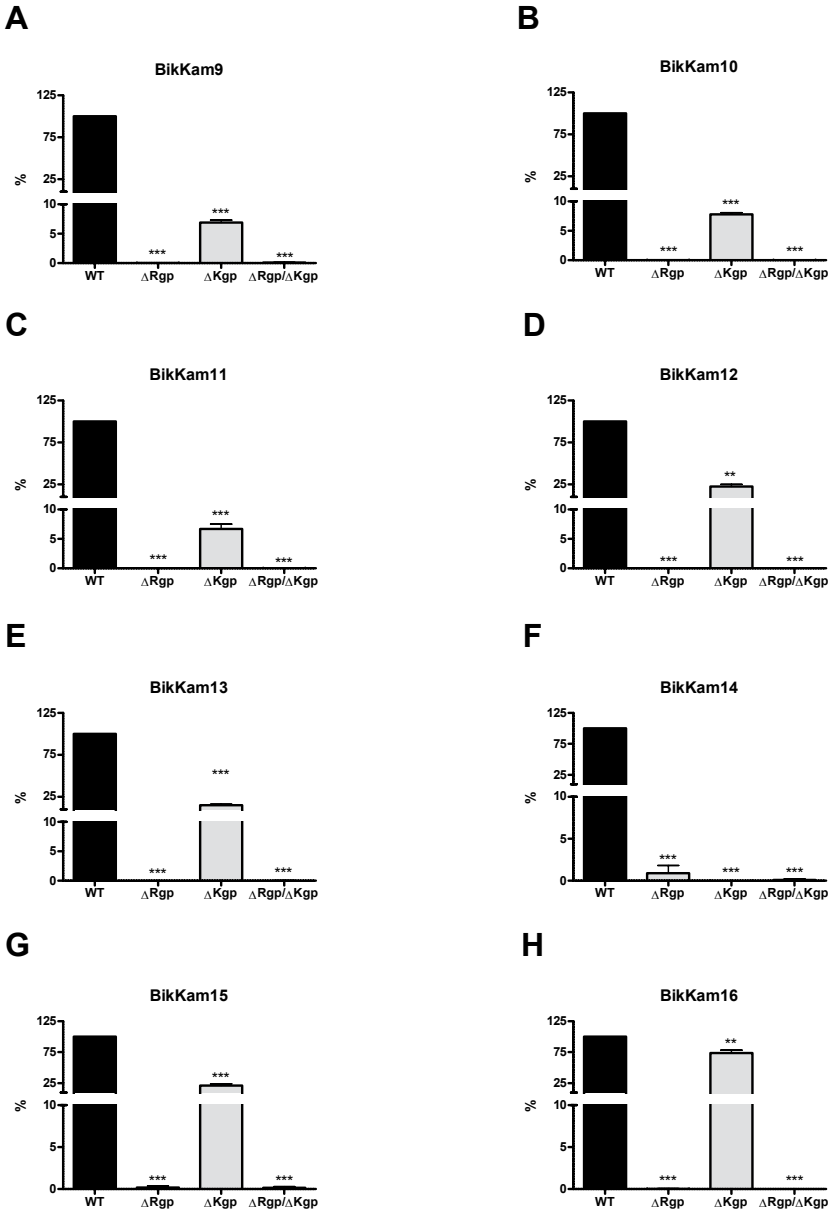

Figure 1. Culture supernatant cleavage activity of gingipain knock-out strains. Culture supernatants of *P. gingivalis* W50 knock-out strains Δ*Rgp* (KDP133), Δ*Kgp* (K1A) and Δ*Kgp*/Δ*Rgp* (KDP136) were incubated with each FRET-substrate (A to H) at 37 ºC. As a positive control, wild-type strain W50 (WT) was used. Cleavage of the substrates was defined in RF/min. The enzyme activity of knock-out strains was compared to *P. gingivalis* W50 activity using the unpaired, two-tailed Student`s *t*-test. Results are expressed as mean ± standard error of the mean (n=3) (***, $P < 0.0001$; **, $P < 0.01$).

minimal concentration *P. gingivalis* that could be detected using BikKam11 to BikKam13 and BikKam15 was 10^7 CFU/ml (Figure 2A). BikKam14 and BikKam15 showed the best sensitivity with a limit of detection of 10^6 CFU/ml (Figure 2A).

To evaluate the possible use of paper points for the collection of clinical samples, paper points were spiked with serial dilutions of *P. gingivalis*. Subsequently, reactivity was tested using the BikKam substrates. It was found that the overall signal was lower compared to the diluted culture (Figure 2B). This had a negative effect on the sensitivity of three of the BikKam substrates. The limit of detection of BikKam13 changed from 10^7 CFU/ml to 10^8 CFU/ml and the detection limit of BikKam 14 and BikKam16 decreased from 10^6 CFU/ml to 10^7 CFU/ml (Figure 2B). Despite the decrease in substrate cleavage, the sensitivity of the other substrates remained unaffected.

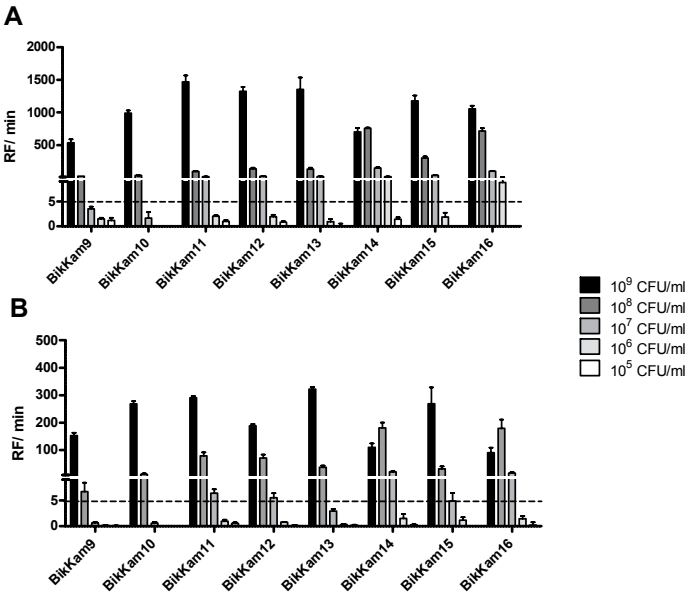

Figure 2. *In vitro* sensitivity of the *P. gingivalis* substrates. Serial dilutions of *P. gingivalis* W50 (A) and *P. gingivalis* spiked paper points (B) were incubated with 16 µM of each FRET-substrate at 37 °C. Enzyme activity was defined in RF/min. The cut-off of the assay was estimated at an RF/min value of 5. Results are expressed as mean ± standard error of the mean (*n*=3).

Screening of patient-derived paper points using *P. gingivalis* substrates

The clinical applicability of the novel protease assay was examined *in situ* by screening 72 paper points obtained from individuals suffering from periodontitis. As a gold

standard the presence of periodontal pathogens was quantified by routine culture (Table S2). Twenty of the paper points were *P. gingivalis* culture positive, varying in *P. gingivalis* concentration from 10^7 to 10^4 CFU/ml. All eluates of the 72 paper points were analysed using all eight substrates. *P. gingivalis* could be detected to a concentration of 10^6 to 10^7 CFU/ml using BikKam9 and BikKam10 and no false-positive activity was observed (Figure 3). BikKam11 to BikKam13 showed a somewhat higher sensitivity, however these substrates were also cleaved by 1 or 2 of the *P. gingivalis* culture-negative samples (Figure 3). The only L-amino-acid-containing substrates (BikKam14 to BikKam16) yielded the highest sensitivity, but the specificity of these substrates was significant lower compared to BikKam9 to BikKam13 (Figure 3).

Earlier, Schmidt and co-workers designed an assay for the detection of gingipain R activity based on the use of chromogenic substrates in which enzyme activity was measured using BANA [39]. Therefore, the commercially available substrate L-BApNA was included in our paper point study and the results were compared to the results obtained using the BikKam substrates. L-BApNA showed a sensitivity for *P. gingivalis* (40%) lower compared to BikKam9 to BikKam13 (60 - 75%) (Table 5). Also the sensitivity of L-BApNA (85%) is lower than that of BikKam9 to BikKam13 (96 – 100%). Overall, the results obtained with our D-amino acid substrates correspond better to the microbiological culture diagnosis. Some of the paper points analysed contained, besides *P. gingivalis*, other oral pathogens such as *P. micros*, *P. intermedia* or *F. nucleatum* subsp. *nucleatum*. No significant correlation was observed between the false positives BikKam9 to BikKam13 and the presence of these other oral pathogens (Table S2).

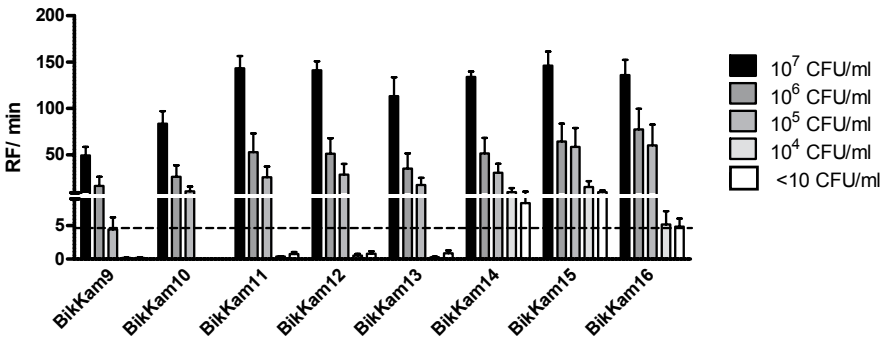

Figure 3. Screening *P. gingivalis* substrates using patient derived paper points. In total 72 patient derived paper points were incubated with 16 µM of each FRET-substrate. Fluorescence was measured for 90 min at 37 °C. Enzyme activity was defined in RF/min. The cut-off of the assay was estimated at an RF/min value of 5. Results are expressed as mean ± standard error of the mean. 10^7 CFU/ml (n=6); 10^6 CFU/ml (n=6); 10^5 CFU/ml (n=5); 10^4 CFU/ml (n=3); < 10 CFU/ml (n=52).

Table 5. Validation of *P. gingivalis* substrates using patient derived paper points

	Sensitivity [a] (%)	Specificity [b] (%)
BikKam9	60	100
BikKam10	60	100
BikKam11	70	98
BikKam12	75	96
BikKam13	70	96
BikKam14	95	64
BikKam15	95	44
BikKam16	95	73
L-BApNA	40	85

[a] Percent BikKam or L-BApNA positive and culture positive
[b] Percent BikKam or L-BApNA negative and culture negative

Discussion

The presence of D-amino acids in fluorogenic protease substrates has proven to be crucial for their specificity. Recently we have successfully shown that substrates containing D-amino acids were specifically cleaved by bacterial proteases, as demonstrated for *Bacillus* spp. [20]. No cleavage was observed in human secretions or fluids such as saliva or serum [20]. To further explore this concept, 115 novel fluorogenic dipeptides were designed, each containing none, one or two D-amino acids. Screening of this library, using culture supernatant of several oral pathogens, resulted in the identification of eight *P. gingivalis*- specific substrates. Five of these substrates indeed contained a D-amino acid (Table 2). Almost all substrates were cleaved by all of the *P. gingivalis* strains tested, the only exception being BikKam9, where no cleavage activity by *P. gingivalis* capsule serotype K2 was observed. The RF/min value of BikKam9 of this strain was, however, very close to the cutoff value of 5. All three substrates in which no D-amino acid was present (BikKam14 to BikKam16) were also cleaved by proteases present in saliva. Potentially, a diversity of hydrolases present in saliva can be responsible of this cleavage. Cathepsins, for instance, are known to recognise and cleave a wide range of peptide bonds, among which Arg-Arg, Lys-Lys and Phe-Arg [6,10,15,22,30,32,32,42]. As well as the cathepsins, other salivary proteases, like alanine aminopeptidase and dipeptidyl peptidase IV, may play a role in the cleavage of these three substrates [2].

In addition to saliva, BikKam15 was also cleaved by the culture supernatant of *T. forsythia.* It is known that *T. forsythia,* similar to *P. gingivalis,* produces secretory prote-

ases [40]. One of these proteases, karilysin, has a high preference for substrates which contain a hydrophobic residue, such as phenylalanine, at the P1 position [21].

The sensitivity for *P. gingivalis* of the all L-amino acid -containing substrates (BikKam14 to BikKam16) was high. However, due to the presence of D-amino acid independent proteolytic activity in saliva, a low specificity was observed (Table 3 and 5). Also, using the clinical samples, a high number of false positives for these substrates were observed (Figure 3 and Table 5). In parallel to BikKam14 to BikKam16, the commercially available L-BApNA also showed a relatively low specificity rate (85%). In contrast, for the D-amino-acid-containing substrates the specificity was high (96 to 100%), strengthening our earlier observation that the use of D-amino acids is crucial in the detection of bacterial proteases using fluorogenic substrates [20].

Despite the increased specificity, the presence of D-amino acids in FRET-peptides had a negative effect on the sensitivity. The substrates which consisted exclusively of L-amino acids were able to detect lower *P. gingivalis* concentrations than the D-amino-acid-containing substrates in culture, as well as in patient samples (Table 3 and 5). The sensitivity of the all-L-amino acid substrates in diluted *P. gingivalis* culture was 10^6 to 10^7 CFU/ml, whereas for the D-amino-acid-containing substrates the observed limit of detection was 10-times higher (10^7 to 10^8 CFU/ml). In general, using the patient samples, more efficient cleavage than that obtained with the diluted *P. gingivalis* culture was observed. Compared to culture, the limit of detection for all substrates was 10-times lower (Figure 2A and Figure 3). This discrepancy might be due to the fact that the pH of the paper point buffer (pH 7.5) is more optimal for gingipain activity than the pH we measured in our *P. gingivalis* cultures, pH 8.5 (data not shown) [12]. Another explanation may be that the amount of gingipains in gingival crevicular fluid is higher, possibly due to the presence of host proteins which might trigger the production of these virulence factors.

Of the 115 substrates from the peptide library only the substrates which contained an arginine or lysine residue were cleaved by *P. gingivalis* culture supernatants. Therefore, we hypothesised that *P. gingivalis*-specific Arg-gingipains and Lys-gingipain are responsible for substrate cleavage [34].

Previously it had been shown that the proteolytic activity of gingipains can be stimulated by the presence of DTT and L-cysteine and inhibited by NEM [5]. The addition of these compounds influenced the proteolysis of all compounds as expected, pointing toward the possible involvement of gingipains (Table 4). It was found that leupeptin inhibited cleavage of all substrates, except for BikKam14, the only substrate in which

no arginine residue is present (Table 4). This finding is in agreement with findings by Aduse-Opoku and co-workers, who observed inhibition of Arg-gingipain activity in the presence of leupeptin, whereas no effect on Lys-gingipain activity was observed [1]. A literature search revealed that the amidolytic activity of both Arg- and Lys-gingipain on L-BApNA is stimulated by the addition of glycyl-glycine [5,7]. In the BikKam substrates however, no amino group is present next to arginine. This can explain the observation that the addition of glycyl-glycine had no effect on the cleavage of our substrates (Table 4).

Experiments using *P. gingivalis* knock-out strains further supported the hypothesis that cleavage of the BikKam substrates is mediated by the gingipains. Absence of these gingpains led to significantly reduced proteolytic activity (Figure 1). In addition to the absence of Arg-gingipain in ΔRgp, it is known that ΔRgp strains secrete Lys-gingipain less efficiently [1,41]. This was confirmed by the decreased proteolytic activity of ΔRgp culture supernatant on the BikKam14 substrate (Figure 1F). We were surprised to measure a reduced cleavage activity of ΔKgp on the arginine containing substrates. We hypothesise that since the Dbc group is coupled to the substrate via the C-terminal lysine side chain, cleavage of the BikKam substrates by Lys-gingipain may take place next to the C-terminal KDbc residue. Further experiments are needed to confirm these assumptions. It has to be noted that all peptides present in the library contain a C-terminal KDbc group. Nevertheless, only the eight substrates described in this study were cleaved by *P. gingivalis*. This clearly points toward the essence of the complete amino acid sequence of the substrate for gingipain recognition and proteolysis. The results obtained strongly suggest a role for the gingipains in the degradation of the Bik-Kam substrates. However, the precise chemical basis for the BikKam cleavage remains to be elucidated.

The use of an enzyme based diagnostic tool based on the presence of *P. gingivalis* Arg-gingipain was previously described by Loesche and co-workers, who assayed plaque samples using L-BApNA [27,28]. They achieved sensitivity similar to that of our test using BikKam11 to BikKam13 (i.e., 6,6 ± 5,8 10^7 CFU/ml versus 10^7 CFU/ml, respectively). The specificity of the BikKam substrates, however, is significant higher at 96 to 98% for the Bikkams versus 85% for L-BApNA. The L-BApNA assay has been developed into a commercially available strip-test which is used as a point-of-care test for *P. gingivalis*, *T. denticola* and *T. forsythia* (BANA test strips, www.oratec.net). Our results imply that the specificity of this rapid and simple diagnostic tool for the detection of *P. gingivalis*

can be improved by the replacement of L-BApNA by one of the D-amino-acid-containing substrates BikKam11, BikKam12 or BikKam13.

Regarding the applicability of these substrates as diagnostic tool for the detection of *P. gingivalis*, their sensitivity, ranging from 10^5 to 10^7 CFU/ml (Figure 3), potentially match the range in which nonsurgical and surgical therapy is unsuccessful and antimicrobial treatment is needed [35]. Consequently, we consider it tempting to hypothesise that the BikKam substrates would not only be helpful in identifying the presence of *P. gingivalis in situ,* but also serve as indicators for antibiotic treatment.

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Supplemental data

Table S1. FRET-peptide substrate library

	Sequence		Sequence		Sequence
PFU-001	FITC-Ahx-A-a-KDbc	PFU-049	FITC-Ahx-H-r-KDbc	PFV-001	FITC-Ahx-c-c-KDbc
PFU-002	FITC-Ahx-G-a-KDbc	PFU-050	FITC-Ahx-K-r-KDbc	PFV-002	FITC-Ahx-d-d-KDbc
PFU-003	FITC-Ahx-S-a-KDbc	PFU-051	FITC-Ahx-P-r-KDbc	PFV-003	FITC-Ahx-e-e-KDbc
PFU-004	FITC-Ahx-V-a-KDbc	PFU-052	FITC-Ahx-R-r-KDbc	PFV-004	FITC-Ahx-f-f-KDbc
PFU-005	FITC-Ahx-C-c-KDbc	PFU-053	FITC-Ahx-W-r-KDbc	PFV-005	FITC-Ahx-h-h-KDbc
PFU-006	FITC-Ahx-D-d-KDbc	PFU-054	FITC-Ahx-Y-r-KDbc	PFV-006	FITC-Ahx-i-i-KDbc
PFU-007	FITC-Ahx-E-d-KDbc	PFU-055	FITC-Ahx-A-s-KDbc	PFV-007	FITC-Ahx-k-k-KDbc
PFU-008	FITC-Ahx-H-d-KDbc	PFU-056	FITC-Ahx-G-s-KDbc	PFV-008	FITC-Ahx-l-l-KDbc
PFU-009	FITC-Ahx-K-d-KDbc	PFU-057	FITC-Ahx-S-s-KDbc	PFV-009	FITC-Ahx-m-m-KDbc
PFU-010	FITC-Ahx-R-d-KDbc	PFU-058	FITC-Ahx-V-s-KDbc	PFV-010	FITC-Ahx-n-n-KDbc
PFU-011	FITC-Ahx-D-e-KDbc	PFU-059	FITC-Ahx-T-t-KDbc	PFV-011	FITC-Ahx-p-p-KDbc
PFU-012	FITC-Ahx-E-e-KDbc	PFU-060	FITC-Ahx-A-v-KDbc	PFV-012	FITC-Ahx-q-q-KDbc
PFU-013	FITC-Ahx-H-e-KDbc	PFU-061	FITC-Ahx-G-v-KDbc	PFV-013	FITC-Ahx-f-r-KDbc
PFU-014	FITC-Ahx-K-e-KDbc	PFU-062	FITC-Ahx-L-v-KDbc	PFV-014	FITC-Ahx-r-r-KDbc
PFU-015	FITC-Ahx-R-e-KDbc	PFU-063	FITC-Ahx-S-v-KDbc	PFV-015	FITC-Ahx-s-s-KDbc
PFU-016	FITC-Ahx-F-f-KDbc	PFU-064	FITC-Ahx-V-v-KDbc	PFV-016	FITC-Ahx-t-t-KDbc
PFU-017	FITC-Ahx-H-f-KDbc	PFU-065	FITC-Ahx-F-w-KDbc	PFV-017	FITC-Ahx-v-v-KDbc
PFU-018	FITC-Ahx-P-f-KDbc	PFU-066	FITC-Ahx-H-w-KDbc	PFV-018	FITC-Ahx-w-w-KDbc
PFU-019	FITC-Ahx-W-f-KDbc	PFU-067	FITC-Ahx-P-w-KDbc	PFV-019	FITC-Ahx-y-y-KDbc
PFU-020	FITC-Ahx-Y-f-KDbc	PFU-068	FITC-Ahx-W-w-KDbc		
PFU-021	FITC-Ahx-D-h-KDbc	PFU-069	FITC-Ahx-Y-w-KDbc		
PFU-022	FITC-Ahx-E-h-KDbc	PFU-070	FITC-Ahx-F-y-KDbc		
PFU-023	FITC-Ahx-F-h-KDbc	PFU-071	FITC-Ahx-H-y-KDbc		
PFU-024	FITC-Ahx-H-h-KDbc	PFU-072	FITC-Ahx-P-y-KDbc		
PFU-025	FITC-Ahx-K-h-KDbc	PFU-073	FITC-Ahx-W-y-KDbc		
PFU-026	FITC-Ahx-P-h-KDbc	PFU-074	FITC-Ahx-Y-y-KDbc		
PFU-027	FITC-Ahx-R-h-KDbc	PFU-075	FITC-Ahx-A-A-KDbc		
PFU-028	FITC-Ahx-W-h-KDbc	PFU-076	FITC-Ahx-C-C-KDbc		
PFU-029	FITC-Ahx-Y-h-KDbc	PFU-077	FITC-Ahx-D-D-KDbc		
PFU-030	FITC-Ahx-I-i-KDbc	PFU-078	FITC-Ahx-E-E-KDbc		
PFU-031	FITC-Ahx-D-k-KDbc	PFU-079	FITC-Ahx-F-F-KDbc		
PFU-032	FITC-Ahx-E-k-KDbc	PFU-080	FITC-Ahx-G-G-KDbc		
PFU-033	FITC-Ahx-H-k-KDbc	PFU-081	FITC-Ahx-H-H-KDbc		
PFU-034	FITC-Ahx-K-k-KDbc	PFU-082	FITC-Ahx-I-I-KDbc		
PFU-035	FITC-Ahx-R-k-KDbc	PFU-083	FITC-Ahx-K-K-KDbc		
PFU-036	FITC-Ahx-G-l-KDbc	PFU-084	FITC-Ahx-L-L-KDbc		
PFU-037	FITC-Ahx-L-l-KDbc	PFU-085	FITC-Ahx-M-M-KDbc		
PFU-038	FITC-Ahx-M-m-KDbc	PFU-086	FITC-Ahx-N-N-KDbc		
PFU-039	FITC-Ahx-N-n-KDbc	PFU-087	FITC-Ahx-P-P-KDbc		
PFU-040	FITC-Ahx-F-p-KDbc	PFU-088	FITC-Ahx-Q-Q-KDbc		
PFU-041	FITC-Ahx-H-p-KDbc	PFU-089	FITC-Ahx-F-R-KDbc		
PFU-042	FITC-Ahx-P-p-KDbc	PFU-090	FITC-Ahx-R-R-KDbc		
PFU-043	FITC-Ahx-W-p-KDbc	PFU-091	FITC-Ahx-S-S-KDbc		
PFU-044	FITC-Ahx-Y-p-KDbc	PFU-092	FITC-Ahx-T-T-KDbc		
PFU-045	FITC-Ahx-Q-q-KDbc	PFU-093	FITC-Ahx-V-V-KDbc		
PFU-046	FITC-Ahx-D-r-KDbc	PFU-094	FITC-Ahx-W-W-KDbc		
PFU-047	FITC-Ahx-E-r-KDbc	PFU-095	FITC-Ahx-Y-Y-KDbc		
PFU-048	FITC-Ahx-F-r-KDbc	PFU-096	FITC-Ahx-a-a-KDbc		

Chapter 4

Comparing culture, real-time PCR and FRET-technology for detection of *Porphyromonas gingivalis* in patients with or without peri-implant infections

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Abstract

The aim of the study was to compare the detection of *Porphyromonas gingivalis* using a fluorescence resonance energy transfer (FRET) technology with commonly used diagnostic methods in salivary and subgingival plaque samples from subjects with dental implants. *P. gingivalis* was considered as a marker for a pathogenic microbiota. Ninety-seven adult subjects were recruited, including periodontally healthy controls with no dental implants, implant controls with no peri-implant disease and patients with peri-implant disease. Saliva and subgingival/ submucosal plaque samples were collected from all subjects and were analysed using culture, real-time PCR and FRET technology employing *P. gingivalis*-specific

substrates. It was found that the *P. gingivalis*-specific substrates were highly suitable for detecting the presence of *P. gingivalis* in saliva and in subgingival plaque samples, showing comparable specificity to culture and real-time PCR. We applied the FRET technology to detect *P. gingivalis* in implant patients with or without an implant condition and in controls without implants. The technique seems suitable for detection of *P. gingivalis* in both plaque and saliva samples. However, with all three techniques, *P. gingivalis* was not very specific for peri-implantitis cases. Future work includes fine-tuning the FRET technology and also includes the development of a chair-side application.

Introduction

Modern comprehensive dental care increasingly includes the consideration of dental implants for patients who need tooth-replacement therapy. For some decades dental implants have served as successful long-term predictable anchors for fixed and removable protheses in fully and partially edentulous patients [1-4]. However, the procedure to replace lost natural teeth with implants offers an unwanted opportunity for bacterial colonization. The early development of biofilms on implant surfaces has been shown to be similar to that on natural teeth and on other restorative materials placed in the oral cavity [5,6]. Over time, from months to years, the microbial population on the implants maturates toward a more complex microbiota [7]. Thus, peri-implant mucosa may also be colonised and infected with increased numbers and proportions of oral bacterial species, analogous to the increase of microorganisms in deep periodontal pockets adjacent to natural teeth.

The inflammatory lesions that develop in the tissue around dental implants are collectively recognised as peri-implant diseases. Manifestation of peri-implant diseases represents a widespread problem. Cross-sectional studies have reported that peri-implant mucositis (i.e. bleeding on probing without concomitant alveolar bone loss) occurs in about 79% of subjects and 50% of mplants [8]. The prevalence of subjects with peri-implantitis varies between 25% and 45% in several publications in accordance with the selected population [8-12].

Studies dealing with the microbiota of failing or failed implants clearly indicate the presence of multiple pathogens that are also associated with periodontitis. Thus, the development of peri-implant diseases appears to be accompanied largely by an increase in specific bacterial species, seemingly similar to those in periodontal diseases. One of the most commonly studied periodontal pathogens [13], and hence a target bacterium in peri-implant diseases, is *Porphyromonas gingivalis* [14]. The presence or absence of *P. gingivalis* in peri-implant sites may be indicative of a pathogenic microbiota, possibly one that is causing peri-implantitis (i.e. infection of tissue and bone around the dental implant).

Methods developed to detect *P. gingivalis* include enzyme assays [15], DNA probe assays [15,16], immunofluorescence [17], anaerobic culture [18] and real-time PCR assays [19,20]. Bacterial culture has long been considered the "gold standard" diagnostic method to detect and quantify the microbiota colonising the oral cavities and periodontal lesions. Moreover, bacterial culture is needed to create an antibiogram. However, not all bacteria can be readily cultured, and the proportional recovery of culturable species is

unlikely to match their proportions in the patient. In addition, preparing cultures is time-consuming and labor-intensive because oral pathogens are often anaerobic and tend to grow slowly. Also, the use of selective media can restrict the growth of many species from periodontal and peri-implant samples. Real-time PCR is a convenient quantitative method that enables the detection of low numbers of cells. However, despite its high sensitivity and specificity, this method does not provide evidence of pathogen viability and bacterial activity.

Bacterial enzymes, such as proteases, are in theory ideally suited as biomarkers for quick and sensitive identification of microorganisms in clinical samples [15]. Many of these enzymes are released into the surrounding microenvironment where they can be detected using sensitive fluorogenic and/or luminogenic substrates [21]. Notably, *P. gingivalis* secretes a variety of proteases that act as virulence factors, thus allowing these bacteria to invade the host tissues. By liberating amino acids from host proteins, secreted proteases are involved in the (anaerobic) metabolism of these microorganisms [22]. Recently, our group has developed fluorogenic substrates (i.e. BikKam substrates) which, due to the presence of D-amino acids in these substrates, appeared highly specific for the detection of bacteria-derived proteases [23,24]. No cleavage of the BikKam substrates has been found by human proteases in various body fluids [23,24]. Using substrates specifically cleaved by *P. gingivalis* proteases (identified as gingipains), we have been able to detect quickly (within minutes) and with high specificity the presence of *P. gingivalis* in subgingival samples without the need for enzyme isolation or sample pretreatment [24]. This offers a potential diagnostic tool for the diagnosis of oral diseases, such as peri-implant infections, where we could use the presence of *P. gingivalis* as a marker for a potentially pathogenic microbiota.

In the present study we compared the practical feasibility of the *P. gingivalis* specific fluorogenic substrates with methods currently used (culture, real-time PCR) to detect *P. gingivalis* in saliva and plaque samples from the peri-implant sites of both diseased patients and healthy individuals. In addition, we tested the hypothesis that the presence of *P. gingivalis in saliva* would be associated with infections at the peri-implant site, thus testing the feasibility of using saliva as diagnostic fluid for peri-implant health status.

Materials and methods

Subject sample

Ninety seven adult subjects (age-range 29-80 years) were recruited for the present study. Data on demographics and smoking habits were collected using a self-reported questionnaire. The study population consisted of periodontal healthy controls with no dental implants, non-diseased implant controls and patients with peri-implant disease referred for diagnosis and treatment of a peri-implant condition to the Departments of Periodontology or Oral Function and Prosthetic Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), or to the Centre for Implantology and Periodontology, Amstelveen, The Netherlands.

The definition and diagnosis of peri-implant diseases was based on the clinical and radiographic criteria described previously [25,26] (i) peri-implant mucositis (presence of inflammation in the mucosa at an implant with no signs of loss of supporting bone); (ii) peri-implantitis (in addition to inflammation in the mucosa, peri-implantitis is characterized by loss of supporting bone > 2 mm as evidenced on new diagnostic radiographs compared to the time point that the implant-suprastructure interface was established).

Controls included subjects who showed no radiographic signs of alveolar bone loss and/or displayed fewer than five pockets ≥ 5 mm concomitant with a clinical attachment level (CAL) ≥ 2 mm, following periodontal measurements assessed within 3 months before sample collections. These subjects could include successfully treated periodontal patients (see below). The presence of gingivitis was not an exclusion criterion for a control.

The clinical parameters assessed included the number of teeth and the number of implants present, pocket probing depth (PPD), gingival recession, and bleeding on probing (BOP) at six different sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual) of each implant/tooth present, excluding third molars. Bleeding of the peri-implant mucosa was scored dichotomously as present or absent upon completion of probing [27]. On the basis of periodontal measurements and dental radiographs (intra-oral bitewing, peri-apical X-rays and/or orthopantomographs), controls were divided into the following three groups; subjects with no implants but potential candidates for implant therapy (C-NI = control, no implant), subjects with an implant placed and loaded ≤ 6 months (C-RI = control with recent implant) and subjects with a steadily healthy implant with a supra-structure and a functional period of at least 6 months (C-SI = control with stable implant).

Exclusion criteria for peri-implant diseased patients and healthy controls were (i) recent history of the presence of any acute infection; (ii) tooth extraction and trauma less than 2 weeks preceding the sample; (iii) systemic antibiotic treatment during the preceding 3 months; (iv) pregnancy; and (v) systemic diseases that might influence the condition of the periodontal tissues and subgingival microflora. Smoking habits were defined as follows: current smokers were participants who smoked at least one cigarette per day, while non-smokers were defined as those who had never smoked or as those who had stopped smoking at least 3 months before the study. A previous history of periodontal disease did not represent an impediment for entry to this study, but it was recorded.

The current study population was made up of five groups as follows (i) peri-implant mucositis group (mucositis, n=20); (ii) peri-implantitis group (implantitis, n=20); (iii) periodontal healthy group with no implant (C-NI, n=19); (iv) non-diseased implant with recent implant placement group (C-RI, n=19); (v) non-diseased implant with a steadily healthy implant for at least 6 months (C-SI, n=19).

The research project was approved by the Medical Ethics Committee of the VU University Medical Center, Amsterdam (3rd February 2011, #2011/022). All volunteers signed an informed consent to participate.

Bacterial sampling

Sampling techniques, as well as sample storage, dispersion and dilutions were performed through routine procedures and established techniques [18,27-30]. In brief, stimulated whole saliva was collected from all volunteers; this was achieved by asking the subjects to chew on a piece of inert paraffin (PARAFILM® M barrier film, VWR International, Amsterdam, The Netherlands) for 5 min after rinsing his/her mouth with saline to remove food residue before sample collection. The peri-implant site with the deepest inflamed pocket was selected for bacterial sampling. For controls the disto-lingual or disto-palatal peri-implant site was selected in the C-RI and C-SI groups, while in the C-NI group, a caries-free gingival sulcus adjacent to the edentulous candidate implant area was selected. After removing supragingival plaque with sterile curettes using coronal strokes starting from the gingival margin, two sterile paper points (Absorbent Points Cell Pk #504 Fine; Henry Schein U.K. Holdings Ltd., Southall, Middlesex, UB2 4AU England) were introduced to the bottom of each peri-implant or periodontal pocket and removed after 10 s. The paper points were transferred to sterile Eppendorf tubes containing 1.5 ml of reduced transport fluid.

After collection, from each of the 97 salivary and 97 subgingival samples was cultured for detection of *P. gingivalis* within 24 h after sampling; the remainder of all samples was were frozen at -20°C until further laboratory analysis.

Culture

Culture of all samples and identification of *P. gingivalis* colonies was performed through routine procedures and established techniques [18,28-30]. In brief, a total of 100 µl of the subgingival plaque and saliva samples was used for culture after 10-fold serial dilution in sterile phosphate-buffered saline. Samples were grown anaerobically (80% N_2, 10% H_2, 10% $C0_2$) at 37 °C on 5% horse blood agar plates (Oxoid no. 2, Oxoid,Basingstoke, UK) enriched with hemin (5 mg/l) and menadione (1 mg/l) for detection of *P. gingivalis. P. gingivalis* was identified on the basis of Gram staining, anaerobic growth, the inability to ferment glucose, the production of indole, and a positive hemagglutination test with 3% sheep erythrocytes. The total number of colony-forming units (CFU) was counted on blood agar plates and the number of *P. gingivalis* colonies was expressed as a percentage of this total number.

Real-time PCR assay

Real-time PCR was performed in our laboratories using established techniques, which are also applied to routine diagnostic procedures for periodontal patient care [19,31,32]. In short, DNA was extracted from a 100 µl sample of subgingival plaque or from a 100 µl sample of saliva using a commercial kit (MagNA Pure LC DNA isolation kit III; Roche Diagnostics, Indianapolis, IN, USA) in MagNA Pure LC (Roche Diagnostics) according to the instructions provided by the manufacturer. Real-time PCR amplification reactions were carried out in a reaction mixture of 20 µl consisting of 4 µl of sample lysate and 16 µl of reaction mixture containing LightCycler PCR mix, PCR water and primers (forward, 5`-GCGCTCAACGTTCAGCC-3`; and reverse, 5`-CACGAATTCCGCCTGC-3`) and the probe (LC610-CACTGAACTCAAGCCCGGCAGTTTCAA-BBQ) for *P. gingivalis*. The conditions for real-time PCR amplification in a LightCycler 480 (Roche Diagnostics) were as follows: initial denaturation at 95 °C for 5 min, 45 amplification cycles (of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 20 s), followed by one cycle of cooling at 40 °C for 15 s.

FRET-technology

Recently, we established the detection of activity of *P. gingivalis* enzymes in both artificial (spiked) oral samples as well as in subgingival plaque samples from periodontal patients using the fluorescence resonance energy transfer (FRET) technology [24]. Therefore, by applying the fluorogenic BikKam substrates specifically tailored for *P. gingivalis* (Table 1) [24], we further explored here the applicability of FRET-technology. Reactions were performed in nonaffinity, black, 96-well plates (Greiner, Recklinghausen, Germany). Enzyme activity in the samples was determined by incubating 16 µM substrate with 45 µl of saliva and 45 µl reduced transport fluid from paper point samples, supplemented with 5 mM L-cysteine at 37°C per each well. *P. gingivalis* culture supernatant was used as a positive control. Plates were on a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories, Offenburg, Germany) with fluorescence readings taken every 3 min at 485 nm excitation and 530 nm emission wavelengths over a 90 min time period. The experiments were run in duplicate and the average result was calculated for use in further statistical analysis. The slope of the relative fluorescence (RF) divided by time (RF/min) defines the enzyme activity. Samples with a RF/min value of ≥ 5 were considered positive for *P. gingivalis,* as described previously [23,24].

The supernatants of the following *P. gingivalis* strains were used as a positive control in this study: W83, X-2, A7A1-28, ATCC 49417, HG1690, HG1691 and 34-4 [24,33]. These *P. gingivalis* strains were cultured on blood agar plates under anaerobic conditions in 5% CO_2 for 48 h at 37°C. Then,single colonies were inoculated in brain heart infusion medium (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with hemin-menadione, at 37 °C under anaerobic conditions. After 24 to 72 h, the cells were pelleted by centrifugation for 10 min at 10,000 x g. Supernatants, containing secreted enzymes, were sterilised by filtration through a 0.22 µM filter (MilliPore UK limited, Watford, U.K.). These *in vitro* samples were used directly or frozen at -20 °C for later use.

Table 1. *P. gingivalis* specific D-amino-acid-containing FRET-substrates

	Sequence
BikKam9	FITC-Arg-D-Asp-KDbc
BikKam10	FITC-Arg-D-Glu-KDbc
BikKam11	FITC-Arg-D-His-KDbc
BikKam12	FITC-Arg-D-Lys-KDbc
BikKam13	FITC-Arg-D-Arg-KDbc

Statistical analysis

Statistical analysis of the data was performed with PASW STATISTICS 18.0 (SPSS Inc., Chicago, IL, U.S.A.). Means, standard deviations, and frequency distributions were calculated. The sensitivity and specificity of the three techniques applied to subgingival plaque and saliva samples were determined using 2 x 2 contingency tables.

Results

Study population

The demographic and clinical characteristics of the subjects included in the study are presented in Table 2. Forty patients (22 women and 18 men; 37–74 years of age) suffering from either perimucositis or peri-implantitis and 57 healthy controls (27 women and 30 men; 29–80 years of age) participated in the current study. The patients in the mucositis, implantitis and C-SI groups were older than those in the C-NI and C-RI groups. Forty-nine women and 48 men participated in the current study. The majority of subjects (85%) were Caucasian. Of the 97 participants, 18% were current smokers, and the highest number of smokers was found in the C-NI group. Almost 30% of the total study population had a previous history of periodontal disease. All the study subjects had an average of at least 15 teeth present, with the averages ranging from a minimum of 15.5 for the implantitis group to a maximum of 24.9 for the C-NI group. For this study population the average number of implants was 2.7. Notably, the C-NI group had no dental implants by definition and served as a reference group with an edentulous area that was potentially suitable for implant therapy. A mean of 2.2 sites with pocket probing depths ± 5 mm was detected for the whole dentition, while 41 (42.3%) sites sampled had pocket probing depth values of ± 5 mm. In general, the patients with peri-implant disease showed the highest mean value for the percentage of bleeding on probing (20.1% and 15.6% for mucositis and implantitis groups, respectively), thus reflecting the inflammatory state of this group of patients.

Prevalence of *P. gingivalis*

Table 3 depicts the prevalence of *P. gingivalis* as detected by anaerobic culture, real-time PCR and FRET for the 97 subgingival plaque samples. When culture and FRET were used as the detection methods, *P. gingivalis* positive samples were detected more often in patients with peri-implant disease than in the healthy control groups. In fact, the

Table 2. Demographic and clinical data for the study population [a]

	Peri-implant diseased patients (*n*=40)			Non-diseased implant patients (*n*=38)	
	Mucositis (*n*=20)	**Implantitis** (*n*=20)	**C-NI** (*n*=19)	**C-RI** (*n*=19)	**C-SI** (*n*=19)
Age (years)	59.0 ± 8.3	57.4 ± 9.1	47.2 ±12.6	53.1 ±10.5	58.7 ± 11.9
Gender					
Female	13 (65%)	9 (45%)	7 (37%)	14 (74%)	6 (32%)
Male	7 (35%)	11 (55%)	12 (37%)	5 (26%)	13 (68%)
Ethnicity					
Caucasian	19 (95%)	16 (70%)	16 (84%)	16 (84%)	18 (95%)
Other	1 (5%)	6 (30%)	3 (16%)	3 (16%)	1 (5%)
Smoking status					
Non-smoker	18 (90%)	16 (80%)	13 (68%)	15 (79%)	17 (90%)
Smoker	2 (10%)	4 (20%)	6 (32%)	4 (21%)	2 (11%)
History of periodontitis	4 (20%)	3 (15%)	11 (58%)	7 (37%)	4 (21%)
Teeth	18.1 ± 8.7	15.5 ± 8.6	24.9 ± 2.2	22.4 ± 4.3	16.7 ± 10
Implants	3.2 ± 2.3	4.7 ± 3.0	0.0	2.1 ± 1.3	3.2 ± 1.8
Sites with PPD ≥ 5 mm					
All teeth	3.2 ± 2.3	2.2 ± 6.3	2.7 ± 2.3	1.7 ± 1.5	0.8 ± 1.1
Sampled sites	11 (55%)	20 (100%)	5 (26%)	1 (5%)	1 (5%)
BOP (%)					
All teeth	20.1 ± 18.5	15.6 ± 13.1	11.9 ± 5.5	13.6 ± 6.5	7.1 ± 5.2
Sampled sites	20 (100%)	20 (100%)	6 (32%)	0 (0%)	0 (0%)

[a] Values represent means ± SD, number (%) of subjects or means ± SD of teeth, implants, sites present. Mucositis, peri-implant mucositis group; Implantitis, peri implantitis group; C-NI, periodontal healthy with no implant group; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 months; PPD, probing pocket depth; BOP, bleeding on probing.

subgingival plaque samples from six out of 20 implantitis patients were culture positive for the presence of *P. gingivalis*. The peri-implant mucositis group also contributed with one *P. gingivalis*-positive sample. Only four (7%) of 57 samples were positive for *P. gingivalis* when C-NI, C-RI and C-SI were grouped together. Similar findings were observed for the FRET assay; a total of five *P. gingivalis*-positive samples were obtained from the patients with peri-implant disease (with four out of five positive samples originating from the implantitis group), while only one sample from the healthy controls was proved to be *P. gingivalis* positive.

A higher prevalence of *P. gingivalis* was found by real-time PCR in samples from the C-NI group than in samples from the other groups. The highest enzyme activity, found by FRET assays, among all subgingival samples was recorded in the implantitis

Table 3. Results for testing the prevalence of *P. gingivalis* in subgingival plaque samples of the selected sites from the various study groups [a]

			Peri-implant diseased patients (*n*=40)		Non-diseased implant patients (*n*=38)		
			Mucositis (*n*=20)	**Implantitis** (*n*=20)	**C-NI** (*n*=19)	**C-RI** (*n*=19)	**C-SI** (*n*=19)
Culture	**Pg prevalence**		1 (5%)	6 (30%)	1 (5%)	1 (5%)	2 (11%)
	Pg count (CFU/ml x 10^6)		0.8	0.24 ± 0.45	0.10	64.0	0.24 ± 0.27
Real-time PCR	**Pg prevalence**		1 (5%)	4 (20%)	6 (32%)	2 (11%)	2 (11%)
	Pg count (CFU/ml x 10^6)		26.0	8.13 ± 9.41	0.49 ± 0.68	8.0 ± 11.3	7.04 ± 9.84
FRET-assay	**Pg prevalence**		1 (5%)	4 (20%)	1 (5%)	0 (0%)	0 (0%)
	Enzyme activity (RF/min)	**BikKam9**	1.24	10.7 ± 15.9	1.3	0	0
		BikKam10	1.67	24.9 ± 38.1	3.8	0	0
		BikKam11	5.65	58.5 ± 38.1	7.7	0	0
		BikKam12	8.24	59.7 ± 74.3	10.8	0	0
		BikKam13	4.85	53.8 ± 77.9	7.6	0	0
Pg positive for culture, RT-PCR and FRET-assay			1	2	1	0	0
Pg positive for culture and RT- PCR			1	3	1	1	1
Pg positive for culture and FRET-assay			1	3	1	0	0
Pg positive for RT- PCR and FRET-assay			1	2	1	0	0

[a] Values represents numbers (%) of subjects *P. gingivalis* positive or mean total CFU/ml x 106 ± SD in case of culture or counts/ml x106 ± SD in case of RT-PCR or relative fluorescence/ min ± SD in case of FRET. Mucositis, peri-implant mucositis group; Implantitis, peri-implantitis group; C-NI, periodontal healthy, no implant group; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 months; CFU, colony-forming unit; Pg, *Porphyromonas gingivalis*. The mean total counts, mean counts of P.g. species and mean of enzyme activity for each substrate were calculated only including Pg positive individuals. Samples with enzyme activity (RF/min) value ≥ 5 were considered positive to the detection of *P. gingivalis* [23,24]

Table 4. Results for testing the prevalence of *P. gingivalis* in salivary samples from the various study groups [a]

			Peri-implant diseased patients (*n*=40)			Non-diseased implant patients (*n*=38)	
			Mucositis (*n*=20)	**Implantitis** (*n*=20)	**C-NI** (*n*=19)	**C-RI** (*n*=19)	**C-SI** (*n*=19)
Culture	**Pg prevalence**		1 (5%)	1 (5%)	1 (5%)	2 (11%)	0 (0%)
	Pg count (CFU/ml x 10^6)		5.9	0.48	0.28	45.2 62.0	0
Real-time PCR	**Pg prevalence**		6 (30%)	4 (20%)	8 (42%)	8 (42%)	4 (20%)
	Pg count (CFU/ml x 10^6)		0.72 ± 0.74	1.76 ± 2.55	0.96 ± 0.96	1.15 ± 0.92	14.52 ± 21.24
FRET-assay	**Pg prevalence**		8 (40%)	6 (30%)	8 (42%)	5 (26%)	7 (37%)
	Enzyme activity (RF/min)	**BikKam9**	5.8 ± 4.2	4.0 ± 2.7	8.1 ± 4.7	4.8 ± 19.1	4.2 ± 2.7
		BikKam10	0.54 ± 1.5	-0.5 ± 1.4	4.1 ± 5.5	-4.8 ± 10.3	1.3 ± 3.1
		BikKam11	5.0 ± 3.9	4.8 ± 5.9	8.7 ± 8.5	-0.8 ± 10.7	4.0 ± 3.1
		BikKam12	3.3 ± 1.4	3.3 ± 3.3	9.2 ± 8.8	15.8 ± 27.0	2.6 ± 1.5
		BikKam13	5.3 ± 2.7	8.6 ± 5.4	1.0 ± 8.8	10.2 ± 11.5	6.3 ± 4.2
Pg positive for culture, RT- PCR and FRET-assay			0	1	1	0	0
Pg positive for culture and RT-PCR			0	1	1	2	0
Pg positive for culture and FRET-assay			0	1	1	0	0
Pg positive for RT-PCR and FRET-assay			2	3	6	2	2

[a] Values represents numbers (%) of subjects *P. gingivalis* positive or mean total CFU/ml x 106 ± SD in case of culture or counts/ml x106 ± SD in case of RT-PCR or relative fluorescence/ min ± SD in case of FRET-assay. Mucositis, peri-implant mucositis group; Implantitis, peri-implantitis group; C-NI, periodontal healthy, no implant group; C-RI, periodontal healthy with recent implant placement; C-SI, steadily healthy implant for at least 6 months; CFU, colony-forming unit; Pg, *Porphyromonas gingivalis*. The mean total counts, mean counts of Pg species and mean of enzyme activity for each substrate were calculated only including Pg positive individuals. Samples with enzyme activity (RF/min) value ≥ 5 were considered positive to the detection of *P. gingivalis* [23,24].

group (Table 3). Interestingly, a *P. gingivalis* positive result was obtained with each of the three different techniques for the same subgingival plaque samples of only four individuals of the total study population.Table 4 shows the prevalence of *P. gingivalis* in saliva samples, as determined by anaerobic culture, real-time PCR and FRET assays. Culture showed the lowest prevalence of *P. gingivalis*-infected samples among all the study groups. For instance, *P. gingivalis* was was not found by bacterial culture in any saliva sample from the 19 C-SI group subjects. In contrast, real-time PCR and the FRET technique seemed more capable of detecting *P. gingivalis* in saliva. In fact, six and four *P. gingivalis*-positive samples were detected by real-time PCR in the mucositis and implantitis groups, respectively, while FRET detected larger numbers of *P. gingivalis*-positive samples, respectively eight (40%) and six (30%) in the mucositis and implantitis groups. A *P. gingivalis*-positive result was achieved for each of the three different techniques in the same salivary sample in only two patients. Positive results for the same sample were obtained more often with real-time PCR and FRET than with other combinations of the three techniques: for example, six out of eight samples were in agreement for the C-NI group.

Comparison of anaerobic culture, real-time PCR and FRET assays.

There were discrepancies between the results obtained with the three techniques for the detection of *P. gingivalis* from subgingival plaque samples (Table 3). In Table 5 we

Table 5. Comparison of the prevalence of *P. gingivalis* in subgingival plaque samples by anaerobic culture and RT-PCR (A), anaerobic culture and FRET-assays (B), and by RT-PCR and FRET-assays (C)

	Positive	Negative	Total
A. Culture vs RT-PCR			
Positive	7	8	15
Negative	3	79	82
Total	10	87	97
B. Culture vs FRET			
Positive	5	0	5
Negative	5	87	92
Total	10	87	97
C. RT-PCR vs FRET			
Positive	4	1	5
Negative	11	81	92
Total	15	82	97

(A): sensitivity = 77% and specificity = 92% (culture as gold standard)
(B): sensitivity = 67% and specificity = 100% (culture as gold standard)
(C): sensitivity = 58% and specificity = 99% (RT-PCR as gold standard)

present the results of the 97 subgingival plaque samples: the calculated sensitivity and specificity were 77% and 92%, respectively, for real-time PCR compared with anaerobic culture (Table 5A). Comparison of bacterial culture and the FRET assay is shown in Table 5B: a specificity of 100% was obtained for analysis of the subgingival plaque samples, but a sensitivity of 67% was obtained with culture serving as gold standard. In Table 5C the results of real-time PCR and FRET methods were compared. Similarly to the results in Table 5B the specificity was close to 100%, but the sensitivity of the FRET assay was 58% for the detection of *P. gingivalis* from subgingival plaque samples around implants or natural teeth, with real-time PCR serving as the gold standard. Comparison of the three methods for detecting *P. gingivalis* in salivary samples is summarized in Table 6. Among the three detection methods, the comparison between culture and real-time PCR gave the highest score for sensitivity and specificity in saliva (83% and 72%, respectively, for real-time PCR compared with anaerobic culture). Comparison between bacterial culture and the FRET assay, shown in Table 6B, yielded a sensitivity of 63% and a specificity of 75%. The number of positive results determined by real-time PCR and the FRET assay, as summarised in Table 6C, yielded sensitivity and specificity values for the FRET analysis, compared with real-time PCR, of 67% and 78%, respectively.

Table 6. Comparison of the prevalence of *P. gingivalis* in salivary samples by anaerobic culture and RT-PCR (A), anaerobic culture and FRET-assays (B), and RT- PCR and FRET-assays (C)

	Positive	**Negative**	**Total**
A. Culture vs RT-PCR			
Positive	4	26	30
Negative	1	66	67
Total	5	92	97
B. Culture vs FRET			
Positive	2	31	33
Negative	3	61	64
Total	5	92	97
C. RT-PCR vs FRET			
Positive	15	19	34
Negative	15	48	63
Total	30	67	97

(A): sensitivity = 83% and specificity = 72% (culture as gold standard)
(B): sensitivity = 63% and specificity = 75% (culture as gold standard)
(C): sensitivity = 67% and specificity = 78% (RT-PCR as gold standard)

Comparison of detection of *P. gingivalis* in saliva and subgingival plaque samples

The correlation of all positive and negative results for the subgingival plaque and salivary samples was further analyzed using the three techniques (Table 7). When real-time PCR was used, 14 out of 15 subjects who harboured *P. gingivalis* in their subgingival plaque samples also had *P. gingivalis* in their saliva (Table 7B). The detection of *P. gingivalis* in saliva reflected its presence also in the subgingival plaque samples for two out of 10 and two out of five patients when culture and FRET technology, respectively, were used as the selected method (Table 7A and 7C). From Table 6 it is clear that real-time PCR gives the highest number (n=15) of *P. gingivalis*-positive subgingival biofilm samples, while FRET assays give the highest number (n=34) of *P. gingivalis*-positive saliva samples compared with anaerobic culture and PCR (n=5 and n=30 positive saliva samples, respectively).

Table 7. Comparison of the prevalence of *P. gingivalis* in subgingival plaque samples with the prevalence in salivary samples by anaerobic culture (A), RT-PCR (B), and FRET-assays (C)

	Positive	**Negative**	**Total**
A. Culture			
Positive	2	3	5
Negative	8	84	92
Total	10	87	97
B. RT-PCR			
Positive	14	16	30
Negative	1	66	67
Total	15	82	97
C. FRET			
Positive	2	32	34
Negative	3	60	63
Total	5	92	97

(A): sensitivity = 56% and specificity = 97% (subgingival plaque samples as gold standard)
(B): sensitivity = 94% and specificity = 84% (subgingival plaque samples as gold standard)
(C): sensitivity = 63% and specificity = 74% (subgingival plaque samples as gold standard)

Discussion

The purpose of the study was to investigate the practical feasibility of detecting *P. gingivalis* from peri-implant sites and saliva of implant patients using specific FRET-substrates in comparison to culture and real-time PCR. In addition, we studied whether salivary presence of *P. gingivalis* could be linked to *P. gingivalis* infections at peri-implant site/pocket samples, thus in fact testing the feasibility of saliva as diagnostic fluid for the

peri-implant health status; in this case *P. gingivalis* would serve as marker bacterium representing a pathogenic microbiota that affects the success rate of implants. The ultimate goal of this study was to form a base for the development of a diagnostic technology that would enable the dental practitioner to perform a chair-side diagnostic salivary test to identify the presence of *P. gingivalis* serving as microbial marker for a peri-implant infection.

Bacterial culture is considered as the "gold standard" diagnostic method of detecting and quantifying the microbiota colonising the oral cavities and to create an antibiogram. Anaerobic culture is specific in its ability to distinguish species. However, it has limitations compared wit real-time PCR; culture is time-consuming and laborious, and it has a relative low level of sensitivity. Real-time PCR is reliable and able to detect low numbers of bacterial cells, but bacterial DNA needs to be extracted and isolated from the sample, and these processes can be costly and laborious. Furthermore, although real-time PCR has been described as a very specific technique [19,34], it is conceivable that cross-reactivity with other (unknown) species may occur as the oral and subgingival microbiota is extensive and diverse [35]. In contrast, the approach using FRET analysis, as presented in this study, is easy to perform and requires fewer experimental steps and less time. Due to the specific character of the BikKam substrates there is no need for time-consuming enzyme pre-enrichment and purification, thus offering the potential for development into a chair-side test. Moreover, the bacteria as a source of the enzymes need not necessarily be viable, as long as there is bacteria-derived enzyme activity.

We found no straightforward correlation among the results obtained using the three techniques: in the present study, five of 97 subgingival plaque samples and 21 of 97 salivary samples were *P. gingivalis* positive (> 10^4 CFU/ml) by real-time PCR but negative by culture. Isolation of *P. gingivalis* was performed on a nonselective medium, which would hamper the detection of small numbers of the microorganism in the presence of a large background of bacterial cells. Besides, an anaerobic environment is difficult to maintain during sample collection. In addition, in contrast to bacterial culture, real-time PCR also detects nonviable bacterial cells present in the sample (i.e. it does not differentiate between intact DNA from viable and nonviable cells). Furthermore, we observed that real-time PCR gave a higher rate of *P. gingivalis* in subgingival plaque samples in samples for the C-NI group than for the other groups. We speculate that this occurred because of the high number of patients with a previous history of periodontal disease in this group. This finding supports the notion that the residual pockets, although receiving treatment, may act as reservoirs of *P. gingivalis*. The highest enzyme activity recorded

in subgingival plaque samples by the FRET assays was obtained for the implantitis group. This suggests that a higher level of *P. gingivalis* is present in peri-implantitis patients. However, the results obtained using the FRET method, which also has the potential to detect viable bacterial cells, did not always match the results obtained by real-time PCR in subgingival plaque samples. In fact, FRET positive/ real-time PCR-negative and FRET-negative/ real-time PCR-positive discrepancies negatively influenced the sensitivity and specificity of the FRET-based technology for the tested oral bacteria, assuming that in these comparisons real-time PCR is truly a gold standard. However, we suggest that for saliva samples, FRET assays may prove to be superior over real-time PCR, given the possibility that bacterial DNA has been degraded in saliva. The inclusion of more and other specific substrates in future research may shed light on this issue.

When culture was used as a gold standard, the FRET method demonstrated in subgingival plaque samples a low sensitivity (67%) as a result of the FRET-positive/ culture-negative ratio. The number of false positives generated by FRET could represent crossreactivity with other bacterial species of which we are not aware and that could produce active proteases specific for the *P. gingivalis* peptides (Table 1) [24]. However, the high specificity (100%) excluded the chance to falsely detect *P. gingivalis* using the FRET technology in subgingival plaque samples. Comparable results were obtained when real-time PCR served as a gold standard for detection of *P. gingivalis* in subgingival plaque samples in comparison with FRET assays (sensitivity = 58%, specificity = 99%). In contrast, the specificity of FRET assays was lower in saliva (specificity = 75% in anaerobic culture and specificity = 78% with real-time PCR as the gold standard). Therefore, once again we need to appreciate that it may be not justified to use culture and real-time PCR as gold standards when analyzing the microbiota of saliva samples; each has its own limitations, as outlined above, and the results are also dependent on the site or the organ sampled. Any new technique for the analysis of the oral microbiota faces the problem of being compared with a "gold standard" that is not a perfect system to start with. Only complete sequencing of the oral microbiome may overcome this to a great extent. However, for this purpose intact bacterial DNA is still needed. Therefore, we feel confident to suggest that, in addition to culture and real-time PCR, *P. gingivalis* can also reliably be detected in saliva by employing the FRET technology. Notwithstanding that the literature seems to support an association between *P. gingivalis* and peri-implant infections, also in studies where paper points were used as a detection method [36], we found that only a limited number of peri-implant pockets in patients suffering from peri-implantitis were positive for the presence of *P. gingivalis*, regardless

of the detection method (six out of 20 by culture, and four out of 20 by real-time PCR or FRET). We postulate two possible explanations. First, the use of paper points might not offer the best method to obtain representative bacterial samples in peri-implant sites or pockets. In many instances the implant suprastructure might prevent proper access to the peri-implant sulci, thus hampering the sampling procedure and leading to erratic results. In addition, the bacterial infection is "hiding" within the screw threads of the implant. Second, *P. gingivalis* is an inadequate bacterial marker for peri-implantitis, based on subgingival plaque sampling. We have to recognize that peri-implantitis is a polymicrobial infection and multiple species could – individually or in combination – be associated with this complication of implant dentistry. Therefore, at this point more marker bacterial species should be included in the study of the complex peri-implant microbiota, and this knowledge will serve for future work expanding FRET technology in saliva. It could be considered a weakness of this study that only one pocket was sampled for each individual and we compared the outcomes with salivary sampling. In fact, when considering the number of subgingival samples needed to detect the presence of *P. gingivalis* in periodontitis patients, selection of the deepest pocket in each quadrant is the most efficient method of sampling [37]. However, in studies on peri-implantitis this is not possible because peri-implantitis is present most often at only one implant. In the current study we used D-amino acids containing substrates that can be used for enzyme-based diagnostic purposes; these substrates appear to be specific for bacterial proteases [23,24]. Even with the current limited set of substrates, promising results have been obtained. More specific D-amino acids containing FRET substrates can be designed for the refinement of *P. gingivalis* sensitivity and the identification of other bacterial species. We suggest that the FRET technique may have special value in salivary bacterial diagnostics and peri-implantitis or perimucositis. Also, the risk for this condition might be an important target. In conclusion, we applied the FRET technique to detect *P. gingivalis* in implant patients with or without an implant condition, and in controls without implants. The FRET technique might also be suitable for detecting *P. gingivalis* in saliva samples: overall, FRET assays showed a higher rate of *P. gingivalis*-positive saliva samples. However, *P. gingivalis* is not very specific for peri-implant cases, as subjects with peri-mucositis and controls can also harbour this species in their saliva. Future work includes fine-tuning the FRET technology, and development of this technology into a chair-side application and multispecies testing. The current pilot study indicates that further investigations into additional D-amino acid substrates and other bacterial markers are warranted to increase the diagnostic strength and applicability.

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Chapter 5

Peptide-based fluorescence resonance energy transfer protease substrates for the detection and diagnosis of *Bacillus* species

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Abstract

We describe the development of a highly specific enzyme-based fluorescence resonance energy transfer (FRET) assay for easy and rapid detection both *in vitro* and *in vivo* of *Bacillus* spp., among which the members of the *B. cereus* group. Synthetic substrates for *B. anthracis* proteases were designed and exposed to secreted enzymes of a broad spectrum of bacterial species. The rational design of the substrates was based on the fact that the presence of D-amino acids in the target is highly specific for bacterial proteases. The designed D-amino acids containing substrates appeared to be specific for *B. anthracis*, but also for several other *Bacillus* spp. and for both vegetative cells and spores. With the use of mass spectrometry (MS), cleavage products of the substrates could be detected in sera of *B. anthracis* infected mice, but not in healthy mice. Due to the presence of mirrored amino acids present in the substrate, the substrates showed high species specificity, and enzyme isolation and purification was redundant. The substrate wherein the D-amino acid was replaced by its L-isomer showed a loss of specificity. In conclusion, with the use of these substrates a rapid tool for detection of *B. anthracis* spores and diagnosis of anthrax infection is at hand. We are the first who present fluorogenic substrates for detection of bacterial proteolytic enzymes that can be directly applied *in situ* by the use of D-oriented amino acids.

Introduction

The Gram-positive *Bacillus anthracis* bacterium is the causative agent of anthrax. The stability, ease of production, and infectious capacity of the spores confer upon *B. anthracis* a high potential as a biological weapon [1]. During pulmonary anthrax, spores germinate in the lungs ultimately followed by the emergence of vegetative anthrax in the circulation [2]. This systemic infection frequently results in secondary shock and multiple organ failure, which, if untreated, results in death [3]. Therefore, fast point-of-care diagnosis is critical for effective treatment of pulmonary anthrax.

B. anthracis possesses two major virulence components; the pXO1 and pXO2 plasmids [2] These plasmids encode the biosynthesis pathway for D-glutamic acid and the anthrax toxins lethal factor (LF), edema factor (EF) and protective antigen (PA), respectively [4-6]. Because of their specificity to *B. anthracis* and their absence in the closely related species *B. cereus* and *B. thuringiensis*, to date the pXO1 and pXO2 plasmids are the main targets used in the detection of *B. anthracis* and the diagnosis of anthrax. For instance, numerous antibody-based detection methods for *B. anthracis* target the pXO1 encoded anthrax toxins [7-9]. Other DNA-based techniques used in the detection of *B. anthracis* are (real-time and multiplex) PCR targeting pXO1 and pXO2 [10]. However, despite their high specificity, these tests do not provide evidence on pathogen viability and disease progression.

Bacterial enzymes, such as proteases, are in theory ideally suited as biomarkers for quick and sensitive identification of microorganisms in clinical samples [11]. Many of these enzymes are released into the surrounding microenvironment and are accessible for detection based on sensitive fluorogenic and/or luminogenic substrates. However, in practice lack of specificity has proven to be a large hurdle, which has seriously hampered practical application for diagnosis [11-13].

Proteases occur abundantly in all organisms, from viruses to men. They are involved in a myriad of processes and functions, from simple digestion of food proteins to highly regulated cascades such as the blood-clotting and the complement cascades [14]. It is therefore obvious that proteolytic activity by itself is not a useful indicator for the presence of bacteria, let alone for a specific pathogen. To overcome this problem, studies have been undertaken to develop substrates with exquisite specificity for protease(s) from a specific pathogen [15-17]. However, the substrates developed thus far still suffer from a lack of specificity, as they can be hydrolysed by a variety of bacterial and human enzymes [14]. For their use as diagnostic tools, therefore, processing of the sample to isolate the target enzyme under investigation is still needed. This is laborious,

time-consuming, and costly, and moreover, prone to yielding erratic results. Multiple enzymatic assays for the detection of *B. anthracis* using the fluorescence resonance energy transfer (FRET-) technology have been described by others [10,17-22]. Most of these assays are based on the detection of the anthrax toxins LF and EF [10,20], Boyer and co-workers studied the LF activity on a 45-amino acid artificial substrate using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [17,18]. When applied to sera of infected monkeys, the assay detected the toxin in the femtomolar range. However, the activity of unrelated proteolytic enzymes will lead to false-positive results, and hence, the detection of LF had to be facilitated by a time-consuming immunoextraction.

In the present study a different approach was developed to enhance the specificity of protease substrates. This approach exploited an intriguing difference between eukaryotic and prokaryotic cells with regard to the use of D-amino acids. While L-amino acids are primarily incorporated in natural proteins, the presence of D-amino acids is highly specific for bacterial proteases, in which they are abundantly present as a component of the bacterial cell walls [23]. This led us to hypothesise that bacteria may express proteases which possess a unique property for recognising and hydrolysing D-amino-acid-containing substrates. Such activities can be translated into diagnostic tests.

The aim of the present study was to develop FRET-based substrates for the specific detection of proteolytic enzymes of the *B. cereus* group which includes *B. anthracis*.

Materials and methods

Bacteria

The bacterial isolates used in this study are *Bacillus anthracis* Vollum (ATCC 14578), *Bacillus cereus*, *Bacillus thuringiensis* var. kurstaki aizawai, *Bacillus globigii*, *Bacillus mycoides* (ATCC 14579), *Bacillus subtilis* Marburg (ATCC 6051), *Bacillus megaterium*, (ATCC 15374), *Yersinia pseudotuberculosis* (ATCC 29833), *Yersinia pestis* HmsF- (ATCC 19428), *Brucella suis* Thomsen, *Brucella melitensis* 16M, *Escherichia coli* (ATCC 11775), *Micrococcus luteus*, *Erwinia herbicola* (ATCC 33243), *Listeria monocytogenes* EGDe (ATCC BAA-679), *Salmonella typhimurium*, *Salmonella montevideo*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus aureus* MRSA (ATCC 43300), *Staphylococcus aureus* MSSA (ATCC 25923), *Acetinobacter lwoffii calcoaceticus* Ruh88, *Vibrio cholerae*, and *Clostridium botulinum* type A (NCTC 2916). Bacteria were grown overnight in 5 ml

of brain heart infusion (BHI) medium (BioTrading, Mijdrecht, The Netherlands) at 35 °C and at 26 °C for the *Yersiniae.* The next day, the bacteria were pelleted by centrifugation for 10 min at 10,000 x g. Supernatant, containing secreted enzymes was sterilised by filtration through a 0.22 µM filter (Millipore, Amsterdam, The Netherlands). The crude samples were used directly or stored at -20 °C for later use.

FRET assay

FRET-substrates were designed by using the MEROPS database [14] and provided by PepScan Presto (Lelystad, The Netherlands) with a purity of approximately 90%. The identity of the substrates was confirmed by mass spectrometry. The substrates were denoted as "BikKams" (Table 1). Assays were performed in black, clear bottom 96-well plates (Corning, Lowell, USA). Enzyme activity in bacterial supernatants was determined by incubating 16 µM substrate with 100 µl of filtered culture supernatant at 37 °C. Filtered BHI medium was used as a negative control. Plates were read with 10 min intervals on a CytoFluor 4000 (Applied Biosystems, Foster City, USA) with excitation at 485 nm and emission at 530 nm. Relative fluorescence (RF) is the value obtained after correction with the negative control; BHI medium. The measured enzyme activity is defined in RF per minute (RF/min).

Table 1. FRET-substrates designed in this study

	Sequence
BikKam1	FITC-Leu-D-Leu-KDbc
BikKam2	FITC-D-Leu-Leu-KDbc
BikKam3	FITC-Leu-Leu-KDbc
BikKam4	FITC-D-Leu-D-Leu-KDbc
BikKam5	FITC-Leu-D-Leu-Leu-KDbc
BikKam6	FITC-Leu-D-Val-KDbc
BikKam7	FITC-Gly-D-Leu-KDbc
BikKam8	FITC-Gly-D-Ala-KDbc

Preparation of spores

B. anthracis strain Vollum (ATCC 14578) and *B. subtilis* strain Marburg were cultured shaking at 35 °C in 250 ml sporulation broth (SB). At a sporulation efficiency of 99% the suspensions were centrifuged at 4,000 x g for 40 min. The pellets were washed with distilled water and resuspended in 1 ml water. The number of viable spores in the suspensions was determined by plating 10-fold serial dilutions on trypticase soy agar

(TSA) plates (BioTrading, Mijdrecht, The Netherlands). Plates were incubated at 35 ºC, and spores were enumerated after 1 day incubation.

Preculture detection of *B. anthracis* spores

B. anthracis and *B. subtilis* spores were precultured in 1 ml BHI medium at 35 ºC. Samples were taken 0, 1, 2, 3 and 4 h after incubation. After centrifugation the samples were incubated using 16 µM of the BikKam1 substrate. The increase in fluorescence was measured for 1 h with 10 min intervals on a CytoFluor 4000 (Applied Biosystems, Foster City, USA) with excitation using a 485 nm filter and emission using a 530 nm filter. Relative fluorescence (RF) is the value obtained after correction with the negative control; BHI medium. The measured enzyme activity is defined in RF per minute (RF/min).

In vivo infection model

Anesthetized male Balb/C mice (Harlan, Horst, The Netherlands) were intranasally inoculated with 1.25 x 10^4 *B. anthracis* spores in a 50 µl volume. For each time point 10 mice were used. At 0, 12 and 48 h postinfection, serum was isolated and pooled per time point. Because of the small amount of sera, samples were analysed using liquid chromatography-electrospray tandem mass spectrometry (LC-ES MS/MS) as described below. During the experiment the animals were kept in sterile isolators (UNO, Zevenaar, The Netherlands) in a biohazard animal unit. They were fed irradiated food (Harlan, Horst, The Netherlands) and acidified water ad libitum. The mice were monitored regularly for clinical status and weighed daily. All experimental procedures performed on the animals were approved by the The Ethical Committee on Animal Experimentation of TNO (DEC 2727).

LC-ES MS/MS

For the LC-ES MS/MS analysis of the infected mouse serum, 1:10 in 0.06 M EDTA diluted serum was incubated with 16 µM FRET-substrate for 3 h at 37 ºC. Assay mixtures were loaded onto an Amicon 10,000 MWCO filter (Millipore, Amsterdam, The Netherlands) that had been preconditioned with 300 µl of 50% acetonitril, and 300 µl of 0.2% (v/v) formic acid in water, respectively. After the sample loading, the filter was washed with 100 µl of 0.2% (v/v) formic acid in water. The collected samples were analysed using LC-ES MS/MS.

LC-ES MS/MS experiments were conducted on a Q-TOF hybrid instrument (Micromass, Altrincham, UK) equipped with a standard Z-spray ES interface (Micromass)

and an Alliance type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a precolumn splitter (type Accurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted, and a PepMap C_{18} column (15 cm 1 mm i.d., 3-µm particles; LC Packings, Amsterdam, The Netherlands).

A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation, as follows: 100% A (at 0 min, 0.6 ml/min flow) to 10% A and 90% B (at 45 min, 0.6 ml/min flow). The flow delivered by the LC equipment was split precolumn to allow a flow of approximately 40 µl/min through the column and into the ES MS interface.

The Q-TOF was operated at a cone voltage of 20-25 V, employing nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 l/h, respectively). MS/MS product ion spectra were recorded using a collision energy of 10-11 eV, with argon as the collision gas (10^{-4} mbar).

Results

Concept and peptide design for a FRET-based anthrax detection method

To design substrates specific for *B. anthracis* we used the MEROPS database to search for known peptidases produced by *B. anthracis.* Peptidases suitable for our purpose should be able to cleave substrates wherein D-oriented amino acids are present to protect the substrate from cleavage by other enzymes. One of the selected enzymes was dipeptidase AC which recognises substrates with the sequence Leu-D-Leu. Besides in *B. anthracis* this peptidase is also produced by several other bacterial species such as *Acinetobacter calcoaceticus* and *Brucella* spp. [14,24]. To check the specificity of the Leu-D-Leu substrate (BikKam1) the substrate was incubated with culture supernatant of numerous bacterial species. It appeared that BikKam1 was only cleaved by culture supernatants of *Bacillus* spp., whereas no cleavage of BikKam1 by culture supernatants of *A. calcoaceticus* or *Brucella* spp. was observed (Table 2). From these results we hypothesised that probably the cleavage of this substrate is not related to dipeptidase AC. To obtain more insight in the mechanism of action variants on the BikKam1 substrate were designed (Table 1). All substrates designed contained fluorescein isothiocyanate (FITC) as probe and Dabcyl (Dbc) as its quencher. The importance of the placement

Table 2. Proteolytic activities of bacterial culture supernatants against the designed FRET-substrates [a]

	Leu-D-Leu	D-Leu-Leu	Leu-Leu	D-Leu-D-Leu	Leu-D-Leu-Leu	Leu-D-Val	Gly-D-Leu	Gly-D-Ala
B. megaterium	++	+	-	+	+	+	+	-
B. anthracis (*B. cereus group*)	++	++	-	-	++	+	++	-
B. cereus (*B. cereus group*)	++	++	-	+	+	+	++	-
B. thuringiensis (*B. cereus group*)	+	+	-	+	+	+	+	-
B. mycoides (*B. cereus group*)	+	+	-	-	+	+	+	-
B. licheniformis	++	++	-	++	++	++	+++	-
B. globigii	-	-	+++	-	-	-	-	-
B. subtilis	-	-	++	-	-	-	-	-
A. calcoaceticus	-	-	+	-	-	-	-	-
B. suis	-	-	-	-	-	-	-	-
B. melitensis	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	-	-	-	-	-	-	-	-
Y. pestis	-	-	-	-	-	-	-	-
V. cholerae	-	-	+++	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-
M. luteus	-	-	-	-	-	-	-	-
E. herbicola	-	-	+++	-	-	-	-	-
L. monocytogenes	-	-	-	-	-	-	-	-
S. typhimurium	-	-	-	-	-	-	-	-
S. montevideo	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	+++	-	-	-	-	+
S. aureus (MRSA)	-	-	-	-	-	-	-	-
S. aureus (MSSA)	-	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	-	-
C. botulinum type A	-	-	+++	-	-	-	-	-
Human serum	-	-	-	-	-	-	-	-
Human saliva	-	-	-	-	-	-	-	-

[a] Enzyme activity is defined in RF/min values as follows: < 5 (-), no activity; 5 to 24 (+), low activity; 25 to 124 (++), moderate activity; > 125 (+++), high activity. Phylogenetic tree is adjusted from Kolsto et al. [25].

of D-leucine was investigated by switching the position of leucine and D-leucine in the sequence (BikKam2). The hypothesis of the specificity of the use of D- amino acids was checked by a substrate in which no D-amino acids are present (BikKam3). BikKam4 was designed to see if the specificity could be increased by the replacement of L-leucine by a D-leucine and in addition to try to increase the cleavage activity an additional L-leucine was added to the Leu-D-Leu sequence (BikKam5). The importance of the presence of leucine in the sequence was investigated by the use of substrates in which one of the D-leucine was replaced by another closely related amino acid D-Val (BikKam6). The substrates Gly-D-Leu (BikKam7) and Gly-D-Ala (BikKam8) were designed by Adachi and co-workers [24] and are both cleaved by dipeptidase AC with a higher efficiency than the Leu-D-Leu substrate.

In vitro evaluation of the BikKam substrates

To further explore the specificity of all the BikKams (Table 1), the substrates were incubated with culture supernatants, potentially containing secreted proteases deriving from a broad spectrum of bacterial species. In all substrates which contain D-leucine, cleavage of the substrate by bacilli of the *B. cereus* group as well as *B. megaterium* and *B. licheniformis* was observed (Table 2). The only exception was the substrate which consists of two D-oriented amino acids (BikKam4). No cleavage of this substrate by *B. anthracis* or *B. mycoides* was observed. All other bacteria tested did not show activity with any of the D-leucine containing substrates (Table 2) including two bacilli which belong to another part of the *Bacillus* tree; the *B. subtilis* group (Table 2). In case D-leucine was replaced by its L-isomer (Bikkam3) a loss in specificity was observed; besides *B. subtilis* and *B. globigii* the substrate was cleaved by culture supernatants of a number of other bacterial species including *Pseudomonas aeruginosa* and *Vibrio cholerae* (Table 2).

To further substantiate the role of the D-leucine in the BikKam1 substrate, D-leucine was replaced by either D-valine (BikKam 6), which structure is closely related to D-leucine, or D-alanine (BikKam8), where the α-carbon atom is covalently bound to a methyl group. BikKam6 was cleaved by all *Bacillus* spp. though with a lower efficiency.

Table 3. Determination of the *B. cereus* and *B. anthracis* BikKam cleavage sites using MS-analysis [a]

	B. cereus			***B. anthracis*** [b]		
BikKam1	FITC-Leu-D-Leu	▼	LysDbc	FITC-Leu-D-Leu	▼	LysDbc
BikKam2	FITC-D-Leu	▼	Leu-LysDbc		n.d.	
BikKam5	FITC-Leu-D-Leu	▼	Leu-LysDbc		n.d.	
BikKam6	FITC-Leu-D-Val	▼	LysDbc	FITC-Leu-D-Val	▼	LysDbc

[a] The cleavage sites of the substrates are denoted with (▼). [b] Not done (n.d.)

No cleavage of the D-alanine substituted substrate by *Bacillus* spp. was observed (Table 2). Replacement of leucine by glycine had no effect on the cleavage pattern or cleavage efficiency. No cleavage activity by *A. calcoaceticus* or *Brucella* spp. was observed on the Gly-D-Leu and Gly-D-Ala substrates (Table 2).

Identification of the *B. anthracis* cleavage sites by LC-ES MS/MS

To identify the cleavage sites, BikKam substrates were incubated with *B. cereus* culture supernatant and analysed using LC-ES MS/MS. All analysed substrates appeared to be cleaved directly after the D-amino acid (Table 3). To verify these cleavage sites for *B. anthracis* the experiment was repeated for two substrates using *B. anthracis* culture

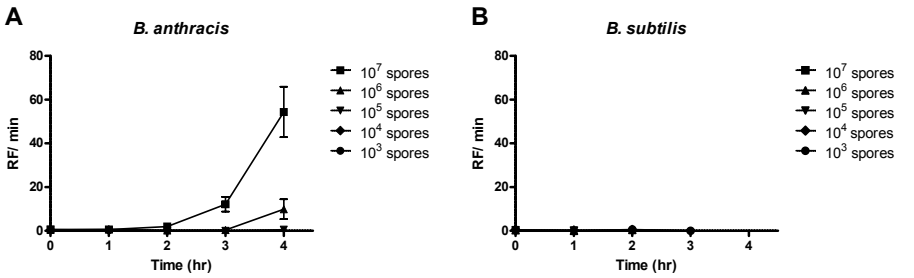

Figure 1. Detection of anthrax spores by BikKam1. Pre-cultivated *B. anthracis* (A) and *B. subtilis* (B) spores were incubated with BikKam1 at 37 °C. After 3 h pre-culturing, 10^7 *B. anthracis* spores, an activity of 11 RF/min with an increase to 87 RF/min after 4 h was measured (■). For the detection of 10^6 *B. anthracis* spores, the spores had to be pre-cultured for 4 h (▲). No increase in fluorescence was observed in case *B. subtilis* spores were used (B). Results are expressed as mean ± standard error of the mean (n=3).

supernatant. As expected *B. anthracis* cleaved the two substrates at the same position as *B. cereus* did.

Detection of precultured *B. anthracis* spores using BikKam1

To verify the applicability of the BikKam1 substrate to detect anthrax spores, different amounts of *B. anthracis* spores were triggered into vegetative state by precultivating and incubated with the substrate. As a negative control spores of *B. subtilis* were used. After 3 h of preculturing 10^7 *B. anthracis* spores, a significant increase of fluorescence was observed (Figure 1A). In case the spores were incubated for 4 h, 10^6 *B. anthracis* spores could be detected. No cleavage was observed when BikKam1 was incubated with precultured *B. subtilis* spores (Figure 1B).

In vivo diagnosis of inhalational anthrax by BikKam1

To further explore the possibilities for the BikKam1 substrate to detect *B. anthracis*, mice were intranasally infected with 1.25 x 10^4 spores/ mouse. Serum was isolated during the infection at 0, 12 and 48 h postinfection (p.i.). BikKam1 cleavage products could be detected in the pooled sera of *B. anthracis* infected mice at 48 h p.i. (Figure 2C). At this time point the mice were very ill and showed onset of severe clinical signs. No cleavage products were found in the sera at 0h (Figure 2A) and 12 h p.i. (Figure 2B).

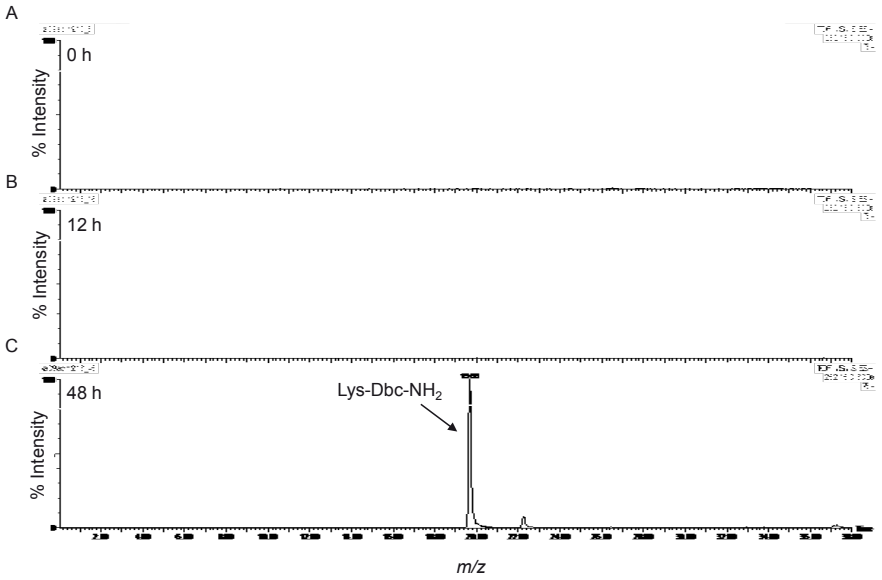

Figure 2. LC-ES MS/MS detection of BikKam1 specific activity in sera of infected mice. Sera taken from *B. anthracis* infected mice were incubated with 16 µM BikKam1 substrate for a 2 h cleavage reaction at 37 °C. At 0 h (A) and 12 h (B) post infection (p.i.) no BikKam1 fragments could be detected. However, at 48 h p.i. a clear peak, identified by MS/MS as the BikKam1 fragment KDbc-NH_2 (MW 396.2), was observed (C).

Discussion

In search for a rapid and simple tool for the detection of bacterial protease activity *in situ*, short and specific substrates containing a D-amino acid were designed. Although L-amino acids represent the vast majority of amino acids found in natural proteins, the presence of D-amino acids is highly specific for bacteria, where D-amino acids are abundantly present as a component of the bacterial cell walls [25]. The fact that bacteria are able to process D-amino acids led us to hypothesise that bacteria may express

proteases which possess a unique property for recognising and hydrolysing D-amino-acid-containing substrates that can be used for detection and diagnostic purposes.

The BikKam1 substrate appeared to be highly specific for the detection of *B. anthracis* and its close relatives (Table 2). No cleavage could be detected in case the substrate was incubated with culture supernatants of *B. subtilis* and *B. globigii* (also known as *B. subtilis* var. niger). These two bacterial species are present in the same branch of the phylogenetic tree of *Bacillus* [26].

To obtain more insight in the mechanism of action, several BikKam1 analogues were designed. The presence of one or more D-oriented amino acids appeared to be important to maintain the specificity of the substrate. Replacement of the D-leucine by its L-isomer (BikKam3) led to a significant decrease in specificity. The BikKam3 substrate was recognised adequately by *B. subtilis* and *B. globigii* proteases, but it was also cleaved by proteases of a number of other pathogens, including *P. aeruginosa* (data not shown). The position of the D-isomer in the substrate seemed to be of limited importance; BikKam1 and BikKam2 were cleaved with similar efficiency. MS-analysis of the cleaved substrates revealed that all analysed BikKams were cleaved directly after the D-oriented amino acid. Both *B. anthracis* as well as *B. cereus* cleaved the substrates at the same position. Changing the position of the D-isomer (BikKam2) or addition of an extra leucine (BikKam5) had no effect on the cleavage pattern. Also in case the D-leucine was substituted by D-valine (BikKam6) the substrate was still cleaved directly after the D-isomer, though it was with lower efficiency. However, substitution of D-leucine by D-alanine (BikKam8) led to a total loss of cleavage activity. This is probably due to the fact that alanine and leucine differ more in structure than valine and leucine do. Both leucine and valine have two methyl groups in their structure, whereas only one methyl group is present in the structure of alanine. Thus, substitution of leucine by alanine has large effects on the steric design of the substrate.

Based on our observations we are tempted to suggest that the BikKam1 substrate might be useful for the detection of *B. anthracis* spores in the so called 'anthrax letters'. For this purpose the spores had to be triggered into a vegetative state by 3 (10^7 spores) or 4 h (10^6 spores) of preculturing to observe a significant increase in fluorescence in time. No increase in fluorescence was observed in case *B. subtilis*, flour, washing powder or talc were used. Currently, culture still is the most common technique to be used in the detection of anthrax [27,28]. The relative ease of this method facilitates a higher throughput compared to immunochemical or PCR methods, which involve multiple and complex sample preparation and assay steps [10,28,29]. One of the methods currently

used in the US Postal Offices is the PCR based technique of Cepheid, with which it is possible to detect 30 anthrax spores in 45 min (http://www.cepheid.com/tests-and-reagents/anthrax/). In contrast, the approach presented in this study is easy to perform, requiring a minimum of experimental steps. Compared to other enzyme based detection techniques it is fast; yet in 4 h 10^7 *B. anthracis* spores can be detected. Due to the specific character of the BikKam1 substrate there is no need for time-consuming enzyme pre-enrichment or purification. We envisage that by using our FRET assay as rapid prescreening "anthrax letters" can be reliably analysed within one day for the presence of *Bacillus* spp. in the field, without the need of highly trained personnel. To confirm the outcome of FRET-mediated testing eventually a *B. anthracis* specific PCR or culturing can be executed in addition.

To explore the opportunities for the assay to diagnose an anthrax infection, sera of *B. anthracis* infected mice were analysed using the BikKam1 substrate. Before infection and 12 h p.i. no BikKam1 degradation fragments could be detected. However, at 48 h after infection BikKam1 fragments could be detected by MS-analysis. At this time-point *B. anthracis* was systemic and the mice were clearly affected by the disease. To detect inhalational anthrax before the onset of severe clinical signs, the infection needs to be diagnosed before the bacterium is present in the vascular system. However, in order to detect *B. anthracis* secreted enzymes in blood the infection probably has to be systemic. In future experiments bronchoalveolar lavage (BAL) fluid will be used to detect anthrax protease activity at an earlier stage.

Further characterization and identification of the enzyme(s) involved will enhance the possibilities to improve the current methodology. In case the responsible enzyme is known, specific stimulators of the assay can be added to the FRET assay to increase the limit of detection. Moreover, the substrate can be optimised by replacement or addition of amino acids or by the usage of other conjugates.

Most likely dipeptidase AC, the enzyme on which the original BikKam1 substrate was originally based, is not involved in cleavage of the in this study used substrates. Instead of dipeptidase AC we hypothesise that the cleavage of the substrate is due to a peptidase which plays a role in cell wall metabolism of *Bacillus* spp., where D-amino acids are incorporated in the peptidoglycan (PGN) [23]. Bacteria release these D-amino acids during their stationary growth phase, probably to synchronize growth inhibition and PGN synthesis [30]. Alternatively, we cannot exclude that either FITC or Dabcyl plays a role in enzyme recognition and thereby sterically hinders dipeptidase AC from *A. calcoaceticus* and *Brucella* spp. from cleaving the BikKam substrates.

A candidate enzyme was recently described by Sela-Abramovich and co-workers who discovered the cysteine peptidase NlpC/p60 (BA1952) [31]. This peptidase is present in sera of anthrax infected mice. Members of the NlpC/p60 family from the genus of *Bacillus* have shown to be DL-endopeptidases that hydrolyse the D-γ-glutamyl-meso-diaminopimelate linkage in the cell wall peptides [32]. Moreover, BA1952 orthologs are present in the secretomes of *Bacillus* spp. of the *cereus* group [31]. However, the BikKam1 substrate was also cleaved by the more remotely related bacilli such as *B. mycoides, B. licheniformis* and *B. megaterium*. Probably, these bacteria produce other enzymes that possess the same functional capacities as the BA1952 peptidase found in *B. anthracis*.

In conclusion, we report a novel enzyme-based approach for the detection of *B. anthracis*. In this study it is shown that the new test can be applied for the detection of *B. anthracis* spores in anthrax letters. Our *in vivo* study in mice might form the basis for *in vivo* diagnosis of *B. anthracis* infection in humans.

We are the first to use D-amino-acid-containing substrates that can be used for enzyme-based diagnostic purposes. We feel it tempting to suggest that, besides *B. anthracis*, the described method can be applied in the detection and diagnosis of other pathogens. Specific D-amino-acid-containing FRET-substrates can be designed for the detection and/or identification of other bacterial species.

Acknowledgements

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Virulence and growth

Chapter 6

Evaluation of a FRET-peptide substrate to predict virulence in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa produces a number of proteases that are associated with virulence and disease progression. A substrate able to detect *P. aeruginosa*-specific proteolytic activity could help to rapidly alert clinicians to the virulence potential of individual *P. aeruginosa* strains. For this purpose we designed a set of *P. aeruginosa*-specific fluorogenic substrates, comprising fluorescence resonance energy transfer (FRET)-labeled peptides, and evaluated their applicability to *P. aeruginosa* virulence in a range of clinical isolates. A FRET-peptide comprising three glycines (3xGly) was found to be specific for the detection of *P. aeruginosa* proteases. Further screening of 97 *P. aeruginosa* clinical isolates showed a wide variation in 3xGly cleavage activity. The absence of 3xGly degradation by a *lasI* knock out strain indicated that 3xGly cleavage by *P. aeruginosa* could be quorum sensing (QS)-related, a hypothesis strengthened by the observation of a strong correlation between 3xGly cleavage, LasA staphylolytic activity and pyocyanin production. Additionally, isolates able to cleave 3xGly were more susceptible to the QS inhibiting antibiotic azithromycin (AZM). In conclusion, we designed and evaluated a 3xGly substrate possibly useful as a simple tool to predict virulence and AZM susceptibility.

Introduction

Pseudomonas aeruginosa, is a Gram-negative rod-shaped bacterium, which is an important cause of infection in individuals suffering from a wide range of underlying disease conditions, including individuals with a compromised host defence, burn patients and in patients suffering from the genetically inherited respiratory tract disease cystic fibrosis (CF). *P. aeruginosa* is also a frequent cause of hospital acquired pneumonia, wound infections and bacteraemia [2]. Early detection of a *P. aeruginosa* infection facilitates effective antimicrobial treatment, reduces inappropriate antibiotic prescription and could possibly contribute to preventing irreversible lung disease in CF patients. Additionally, this bacterial pathogen produces a number of protease enzymes that are associated with virulence and disease progression [16]. In this respect, a substrate able to detect *P. aeruginosa*-specific proteolytic activity could help to rapidly alert clinicians to the virulence potential of individual *P. aeruginosa* strains isolated from different patients. Specifically, detection of these protease virulence factors could potentially be used to monitor the severity of infection and to predict disease outcome, thereby providing useful information to support tailor-made patient monitoring and treatment, a first step towards introducing "personalized medicine".

In theory, bacterial proteases may be suited as biomarkers for the rapid and sensitive identification of microorganisms in clinical samples. Further, the ability to utilize protease based detection methods to diagnose bacterial infections has been previously described [17,28]. In this respect, it is known that *P. aeruginosa* is equipped with a large arsenal of virulence factors that aid to successfully infect the host [16]. The majority of these virulence factors include proteases produced under control of the Las and Rhl quorum sensing (QS) systems of *P. aeruginosa* [11], examples of which include LasA and LasB elastases and alkaline protease [3]. These proteases play an important role in *P. aeruginosa* pathogenesis through the degradation of biologically active proteins present in human tissue [1]. In fact, the significant role of the QS-system in *P. aeruginosa*-related disease progression has resulted in much research into QS-inhibiting compounds that could potentially reduce the organism's virulence potential [5,12,14,15,26]. Further, the therapeutic efficacy of this anti-QS, anti-virulence, strategy in the treatment of *P. aeruginosa* has already been demonstrated for the QS-inhibiting antibiotic azithromycin [26]. Though anti-virulence treatment based on QS-inhibition is only useful when an active, virulence factor secreting, QS-system is present in the *P. aeruginosa* strain to be targetted.

In this study we describe the design of a novel *P. aeruginosa*-specific Fluorescence Resonance Energy Transfer (FRET)-peptide substrate and examine its applicability for the detection of *P. aeruginosa* proteolytic activity in clinical specimens. In addition, we studied the link between substrate cleavage efficiency, virulence factor production and susceptibility towards QS-inhibiting antibiotics, such as azithromycin.

Material and methods

Bacteria

The *P. aeruginosa* strains used for substrate specificity testing are listed in Table 1. Clinical strains of *P. aeruginosa* were collected from a bacterial biobank present within the Department of Medical Microbiology and Infectious Diseases of the Erasmus Medical Center, Rotterdam, Netherlands, collected between the years 2008-2012. In total 97 clinical isolates were selected; 13 from blood, 56 from sputum (35 CF patients and 21 non-CF patients), and 28 strains were isolated from wounds. The VITEK 2 system (bio-Mérieux, Marcy L`Etoile, France) was used for identification and antibiotic susceptibility testing of the clinical isolates. For the preparation of culture supernatants, all bacteria were grown in 5 ml brain heart infusion (BHI) medium (BioTrading, Mijdrecht, The Netherlands) at 37 ^{o}C. After 16 h of culture, the bacteria were pelleted by centrifugation

Table 1. Bacterial strains used in this study

Strain	
Pseudomonas aeruginosa	ATCC 15692, PAO1
Pseudomonas aeruginosa	PA14
Pseudomonas aeruginosa	PA14Δ*lasI* [28]
Pseudomonas fluorescens	Clinical isolate
Pseudomonas putida	S12
Pseudomonas stutzeri	DSMZ 10701, JM300
Staphylococcus aureus	ATCC 43300
Staphylococcus simulans	ATCC 27851
Staphylococcus capitis subsp. *capitis*	ATCC 35661
Staphylococcus epidermidis	Clinical isolate
Streptococcus pneumoniae	ATCC 49619
Streptococcus equi subsp. *zooepidemicus*	ATCC 43079
Klebsiella pneumoniae	ATCC 43816
Haemophilus influenzae	ATCC 49247

for 10 min at 3,000 x g, and the enzyme containing supernatant was filter sterilized through a 0.22 µm filter (Millipore, Amsterdam, The Netherlands).

FRET- assay

The substrates used in this study were purchased at PepScan Presto B.V. (Lelystad, The Netherlands) with a purity > 90%. All substrates were C-terminally flanked with a fluorescent probe; FITC and N-terminally flanked with a lysine coupled quencher; Dabcyl. Identity of the substrates was confirmed by PepScan Presto B.V. using mass spectrometry. Assays were performed in black, clear bottom 96-well plates (Corning, Lowell, USA). Proteolytic activity was determined by incubating 16 µM substrate with 50 µl filtered bacterial culture supernatant or 50 µl lysostaphin (0.04 µg/µl diluted in BHI, Sigma, Zwijndrecht, The Netherlands) at 37 ºC. Filtered BHI medium was used as a negative control. Plates were read for 60 min with 2 min intervals using a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories, Offenburg, Germany) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Relative fluorescence (RF) values were obtained after correction against an un-inoculated BHI culture medium control. Protease activity was defined in RF per minute (RF/min).

Sensitivity testing of the 3xGly substrate *in vitro*

P. aeruginosa PAO1 was cultured overnight in BHI medium at 37 °C. Next day, 20 µl of the overnight culture was added to 20 ml fresh BHI medium. Subsequently, bacteria were grown for another 16 h and a sample was taken every hour. The number of bacteria in the sample was determined by colony counting by plating 10-fold serial dilutions on trypticase soy agar (TSA) plates (BioTrading, Mijdrecht, The Netherlands). Plates were incubated at 37 ºC and bacteria were enumerated after overnight incubation. Proteolytic activity on the 3xGly substrate was examined using 50 µl samples in the FRET-assay as described above. Relative fluorescence (RF) values were obtained after correction against negative culture medium samples. The protease activity was defined in RF per minute (RF/min). Proteolytic activity with an RF/min > 5 was defined positive.

Quantification of pyocyanin production and LasA protease activity

Extracellular pyocyanin production was determined as previously described [9]. LasA protease activity was examined by measuring the ability of stationary phase *P. aeruginosa* culture supernatants (see FRET-assay section above) to lyse heat-killed *Staphylococcus aureus* cells as described by Kessler et al [20].

RNA isolation and cDNA synthesis

A selection of 12 *P. aeruginosa* isolates were chosen for RNA isolation. These isolates had been cultured from blood, wound and sputum clinical specimens, with two isolates that were 3xGly active (+, RF/min > 5) and two isolates that were 3xGly inactive (-, RF/min < 5), being chosen from each clinical specimen type. *P. aeruginosa* strain PAO1 (ATCC 15692) was used as a 3xGly positive reference control. The 12 *P. aeruginosa* isolates were initially grown overnight in BHI medium at 37 °C. Next day the overnight culture was diluted 1:10 in BHI medium and the bacteria again cultured at 37 °C until an OD_{600} of approximately 0.9 – 1.0 was reached. From these cultures total RNA was extracted using the FastRNA ProBlue kit (Promega, Leiden, The Netherlands) according to manufacturer's instructions. To remove any contaminating DNA, 1 µg of extracted RNA was mixed with one unit DNase (Fermentas, Thermo Fisher, Landsmeer, The Netherlands) in 1x DNase reaction buffer. The mixture was then incubated at 37 °C for 30 min and the reaction stopped by the addition of 1 µl 50 mM EDTA and subsequent incubation for 10 min at 65 °C. For cDNA synthesis, DNase treated RNA (500 ng) was incubated for 60 min at 42 °C with 5 µM random hexamer primers, 10 units Ribolock, 1 mM dNTPs and 200 units RevertAid in 1x reaction buffer (Fermentas, Thermo Fisher, Landsmeer, The Netherlands). The reaction was stopped by incubation at 70 °C for 5 min.

Expression of quorum sensing genes

The detection of QS-gene expression was performed using real-time PCR amplification and specifically designed primers for the QS-related pathway genes *lasI* and *rhlA*, the non-QS related control gene *trpD* and the *P. aeruginosa* housekeeping gene *rpsL* [22]. Each PCR contained 1x FastStart SYBR Green mastermix, 4 mM $MgCl_2$ and 0.5 pmol of each primer and 2 µl of 10x diluted cDNA. Amplification was performed using the LightCycler (Roche, Woerden, The Netherlands) The cycling parameters used were: 15 min at 95 °C, 40 amplification cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s. Meltcurve analysis, performed at the end of amplification, showed a single product peak, indicating that no non-specific products were amplified. Data were analyzed using LightCycler software (Version 3.0) and the housekeeping gene *rpsL* was used as a reference (housekeeping) gene for normalizing gene expression. The $2^{-\Delta\Delta Ct}$ method was used to calculate the expression of the genes of interest [23]. QS-gene expression was calculated as a relative percentage of the *rpsL*-normalized gene expression in the control *P. aeruginosa* PAO1 isolate.

Azithromycin susceptibility testing

The susceptibility of *P. aeruginosa* to the QS inhibiting antibiotic azithromycin (AZM) was evaluated for 35 *P. aeruginosa* strains isolated from the sputa of CF-patients using the disc diffusion method. For this purpose, a suspension of each bacterial isolate, overnight grown on tryptic soy agar (TSA) blood agar, was prepared in physiological saline at a turbidity of 0.5 McFarland units. Each bacterial suspension was streaked onto TSA plates with a sterile cotton swab, to obtain uniform bacterial growth, and a disc containing 15 µg AZM (Oxoid, Badhoevedorp, The Netherlands) was placed on the middle of the inoculated culture plate. Plates were then incubated at 37 ^{o}C, incubated overnight and the diameter of the zones of growth inhibition (mm) were measured.

Results

Design of *P. aeruginosa*- specific FRET-substrates

In a study by Vessillier et al. it was described that the *P. aeruginosa* specific protease LasA, recognizes and degrades glycine bonds [27]. Based on these cleavage characteristics we designed FRET-peptide substrates, comprising multiple glycine residues, and incubated these substrates with *P. aeruginosa* culture supernatant. These substrates included FITC-Gly-LysDbc (1xGly), FITC-$(Gly)_2$-LysDbc (2xGly), FITC-$(Gly)_3$-LysDbc (3xGly), FITC-$(Gly)_4$-LysDbc (4xGly) and FITC-$(Gly)_5$-LysDbc (5xGly). As a specificity control, we compared the activity of *P. aeruginosa* culture supernatant towards these substrates with cleavage activity with lysostaphin, an enzyme produced by *Staphylococcus simulans*, that recognizes and degrades penta-glycine bonds [29]. Experiments showed that the 5xGly substrate was cleaved by both lysostaphin and *P. aeruginosa* culture supernatant, and that a similar result was observed for the substrate containing four glycines (Figure 1). However, when the 3xGly substrate was tested, a significant decrease in proteolytic activity by lysostaphin was observed, whereas the proteolytic activity of *P. aeruginosa* actually significantly increased. Additional experiments showed minimal cleavage in case of the 2xGly substrate and no cleavage using the 1xGly substrate. These results indicated that the 3xGly substrate was the best candidate, amongst the substrates tested, to investigate as a potential substrate for the rapid detection of virulence in *P. aeruginosa*.

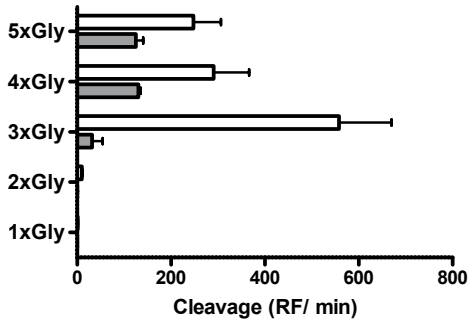

Figure 1. Cleavage activity of lysostaphin and *P. aeruginosa* culture supernatant on a range of glycine substrates. Lysostaphin (2 µg, grey bar) and culture supernatant of *P. aeruginosa* PAO1 (white bar) were incubated with 16 µM FRET- substrate at 37 °C for 1 h. Cleavage of the substrates was defined in RF/min. Results are expressed as mean ± standard error of the mean (*n*=3).

Characterization of 3xGly substrate cleavage

To characterize the specificity of the 3xGly substrate, we examined 3xGly cleavage using a range of culture supernatants (i) from a range of *Pseudomonas* non-*aeruginosa* species, and (ii) from a range of additional respiratory bacteria, and microorganisms known to produce lysostaphin (-like) proteases (Table 1). Results showed that the 3xGly substrate was exclusively cleaved by culture supernatants of *P. aeruginosa* (strains PA14 and PAO1), but not by *P. putida*, *P. stutzeri* or *P. fluorescens*. Further, no cleavage activity was observed for the range of additional respiratory bacteria, and microorganisms known to produce lysostaphin, that were tested. Interestingly, no cleavage activity was observed when the 3xGly substrate was incubated with culture supernatant of the *P. aeruginosa* strain PA14Δ*lasI* which lacks the *lasI* QS-gene (Figure 2). Besides the

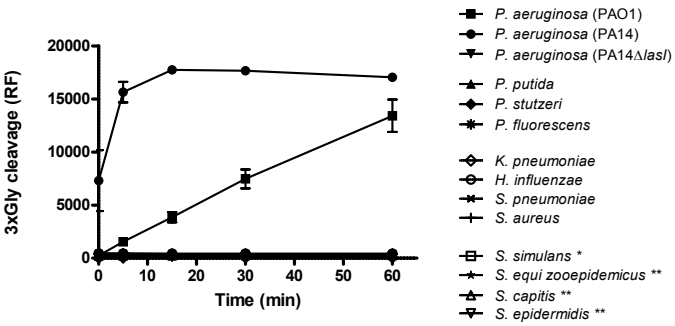

Figure 2. Specificity testing of the 3xGly substrate. Culture supernatants of *Pseudomonas* spp., respiratory micro-organisms and bacteria producing lysostaphin (*) or lysostaphin-like (**) proteases were incubated with 16 µM 3xGly. Fluorescence was measured for 1 h at 37 °C. Results are expressed as mean ± standard error of the mean (*n*=3).

above mentioned bacteria the 3xGly substrate was screened with an additional set of in total 17 bacterial supernatants. None of these bacteria was able to cleave the substrate (data not shown). To characterize 3xGly sensitivity, the substrate was incubated with a dilution series of *P. aeruginosa* PAO1. Using a cut-off of RF/min > 5, it was observed that the limit of detection for *P. aeruginosa* PAO1 using the 3xGly substrate was 10^7 CFU//ml (Figure 3).

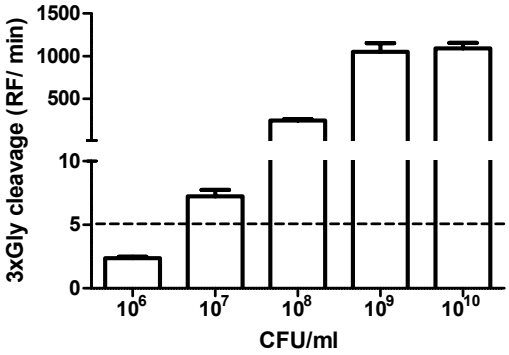

Figure 3. Sensitivity testing of the 3xGly substrate. Serial dilutions of *P. aeruginosa* PAO1 were incubated with 16 µM 3xGly at 37 °C. Cleavage activity was defined in Relative Fluorescence per minute (RF/min). The cut-off of the assay was estimated at an RF/min value of 5. Results are expressed as mean ± standard error of the mean (*n*=3).

In order to obtain a more comprehensive view of protease activity in an extended range of clinically relevant *P. aeruginosa* isolates, we tested a total of 97 randomly selected *P. aeruginosa* clinical isolates that had been cultured from wounds, blood and sputum. These strains were analyzed for their supernatant cleavage activity on the 3xGly substrate. Results revealed that a large percentage of the strains tested (60/97; 62%) were unable to cleave the 3xGly substrate. This percentage varied between clinical specimens from 50% in wound isolates to 73% in isolates from CF patients (Table 2).

Table 2. 3xGly cleavage activity among 97 randomly selected *P. aeruginosa* clinical isolates [a]

			Sputum (*n*=56)	
	Wound (*n*=28)	**Blood** (*n*=13)	**CF** (*n*=35)	**Non-CF** (*n*=21)
Cleavage	14 (50)	4 (31)	9 (27)	10 (48)
No cleavage	14 (50)	9 (69)	26 (73)	11 (52)

[a] Number in brackets denotes percentage (%)

Relationship between 3xGly cleavage and virulence

LasA is a member of the beta-lytic endopeptidase family and pyocyanin is a secondary metabolite which has the ability to oxidize and reduce other molecules [19,24]. The expression of both of these virulence factors by *P. aeruginosa* has been shown to be related to its QS system [16]. Results from both LasA and pyocyanin production indicated that both were significantly higher in 3xGly cleaving *P. aeruginosa* isolates, though some variation in individual isolates was observed (Figure 4A-B). The association between LasA activity and 3xGly cleavage was very significant ($P < 0.0001$, $r = 0.542$).

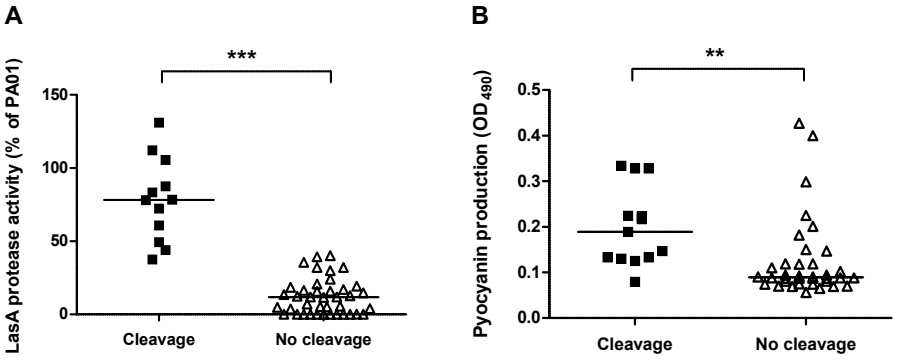

Figure 4. Link between 3xGly cleavage and secretion of QS-related proteins. Culture supernatants of 56 *P. aeruginosa* strains isolated from sputum were analyzed for LasA protease activity (A) and pyocyanin production (B). QS-related LasA cleavage activity in 3xGly cleaving *P. aeruginosa* strains (RF/min > 5) was compared to LasA cleavage in 3xGly non-cleaving strains (RF/min < 5) using the unpaired, two-tailed Students *t*-test. (** $P < 0.01$; *** $P < 0.0001$). A horizontal line indicates the median of LasA activity or pyocyanin production.

Relationship between 3xGly cleavage and the quorum sensing system

The observation of a lack of 3xGly activity using a *P. aeruginosa lasI* knock-out mutant strain (Figure 1) indicated that an absence of 3xGly cleavage activity might be related to an impaired QS-system expression. In order to verify this hypothesis we investigated the expression of the QS-genes *lasI* and *rhlA* using a selection of 3xGly cleaving and non-cleaving *P. aeruginosa* isolates. The strains which lacked 3xGly proteolytic activity showed a reduced expression of *lasI* and *rhlA* when compared to *lasI* and *rhlA* expression in 3xGly cleaving isolates. A significant reduction in normalized *lasI*, but not *rhlA,* was observed ($P = 0.05$). There was no difference observed in the normalized expression of the *trpD* control, a gene unrelated to the QS-system (Figure 5).

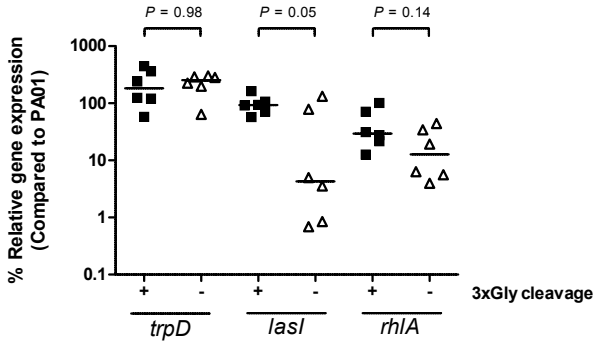

Figure 5. Link between 3xGly cleavage and expression of QS-genes. QS-gene expression was determined in a subset *P. aeruginosa* strains as described in material and methods. Normalized expression of the QS-circuit gene *lasI*, QS-target gene *rhlA* and the QS-independent gene *trpD* measured in mid-log phase grown bacteria is shown as relative values (%) compared to the *P. aeruginosa* PAO1 control strain. A horizontal line indicates the median expression levels. *P*- values were calculated using unpaired, two-tailed Students *t*-tests

Relationship between 3xGly cleavage and azithromycin susceptibility

The apparent association between 3xGly cleavage and the QS-system led us to investigate a possible relationship between 3xGly cleavage and susceptibility to the QS-inhibiting antibiotic AZM. We examined the AZM susceptibility of *P. aeruginosa* clinical isolates using a disc diffusion assay, but found no relationship between 3xGly cleavage and AZM susceptibility for *P. aeruginosa* isolates cultured from blood and wounds, or from the sputum of non-CF patients. Interestingly however, a highly significant association was observed between AZM inhibition zone and 3xGly cleavage for *P. aeruginosa*

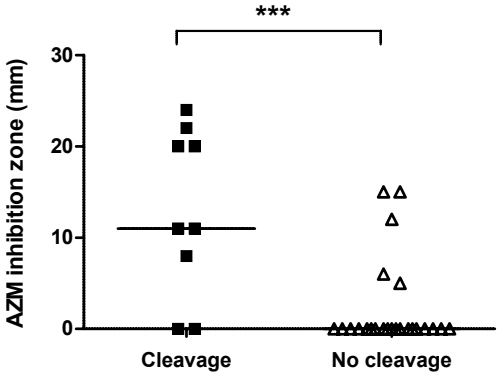

Figure 6. Link between 3xGly cleavage and azithromycin susceptibility. Azithromycin (AZM) susceptibility of *P. aeruginosa* isolates cultured from sputum was determined using the disc diffusion method. AZM inhibition zones for 3xGly cleaving (RF/min > 5) *P. aeruginosa* strains was compared to inhibition zones of 3xGly non-cleaving strains (RF/min < 5) using the unpaired, two-tailed Students *t*-test (*** $P < 0.0001$). A horizontal line indicates the median AZM inhibition zone size.

isolates cultured from the sputa of CF patients (Figure 6). Finally, 3xGly cleavage in relation to a range of AZM unrelated antibiotic resistances was investigated, with no significant correlation being found between 3xGly cleavage and resistance to the other antibiotics tested. However, it was noted that among the group of *P. aeruginosa* isolates that lacked 3xGly cleavage more multi drug resistant (MDR) isolates were observed (Supplemental Table S1).

Discussion

We designed and evaluated a fluorogenic substrate as a potential marker of virulence in the bacterial pathogen *P. aeruginosa.* Preliminary experiments indicated that this 3xGly substrate was specific for cleavage by *P. aeruginosa*, and that the sensitivity of the substrate was 10^7 CFU/ml within 1 h, though this limit of detection may vary among different *P. aeruginosa* strains (it was observed that PA14 cleaved the 3xGly substrate more efficient than PAO1). Only slight cross reactivity was observed with another bacterial protease, lysostaphin, which recognizes and degrades pentaglycin bonds [29]. Later experiments using a broader range of 97 clinical isolates showed that 3xGly cleavage activity differed between *P. aeruginosa* isolates, with a large percentage of the isolates being unable to cleave the 3xGly substrate. This is possibly related to difference in expression of the *P. aeruginosa* QS-system.

One of the most important proteases secreted under the direction of the *P. aeruginosa* QS system is the LasA protease. The LasA protease (staphylolysin) of *P. aeruginosa* can recognize and degrade glycine bonds, degrade elastin and is an important contributor to the pathogenesis of this organism. LasA (20 kDa) is a member of the beta-lytic endopeptidase family of extracellular bacterial proteases, and possesses high-level staphylolytic activity [1]. Further, Elston et al. showed that the LasA protease is capable of the degradation of peptides in which three glycines were present [8]. Therefore, based on the already established glycine-cleaving proteolytic activity of the *P. aeruginosa* LasA protease, its staphylolytic activity, its control via the *P. aeruginosa* QS system*,* and the results observed for 3xGly cleavage, it appears that the LasA protease is most likely the protease virulence factor measured in *P. aeruginosa* culture supernatant using our 3xGly substrate.

Other factors, dependent on the expression of the QS-system of *P. aeruginosa* are pyocyanin production and antibiotic resistance. Pyocyanin is an important virulence factor and functions as an electron transfer facilitator [16]. In addition, *P. aeruginosa*

isolates which lack production of QS-dependent virulence factors, have previously been shown to possess a higher resistance rate to antimicrobials, among which ciprofloxacin and tobramycin [18]. Although, we indeed observed a significant correlation between pyocyanin production and 3xGly cleavage, no significant correlation with resistance to the antimicrobials examined was found (Supplemental Table 1). This discrepancy might be due to difference in culture conditions, as the secretion of QS-metabolites, such as the LasA protease, depends on the availability of nutrients in the environment [7].

Perhaps one of the most interesting findings of the study was the fact that the highest percentage of 3xGly non-cleaving isolates was observed in group of CF sputum isolates. This is possibly due to the fact that CF patients are often colonized with *P. aeruginosa* [10]. Kohler et al showed that during *P. aeruginosa* colonization of intubated patients the number of QS- mutants increases. These mutants lack the production of QS-dependent proteins, such as elastase and rhamnolipids, and take advantage of the "public goods" produced by wild-type isolates that reside within the total population of *P. aeruginosa* isolates that colonize the patient ("cheater" strains). This phenomenon may result in overgrowth of the wild-type *P. aeruginosa* isolate by the mutant isolate due to its fitness benefit [21].

Currently, research is being performed to investigate the potential of QS-inhibiting compounds in the treatment of *P. aeruginosa* related infections [5,12,14,15,26]. The most thoroughly investigated QS-inhibiting antibiotic is AZM. AZM treatment is associated with improvement in disease outcome in *P. aeruginosa* infected CF-patients [25]. However, an anti-virulence agent such as AZM is only effective when an active, virulence factor secreting, QS-system is present. For this reason Kohler et al screened for rhamnolipid production to select patients for their clinical trial on AZM efficiency. Patients infected with *P. aeruginosa* strains which are unable to produce these QS-proteins were excluded from the protocol [26].

We observed a strong correlation between AZM susceptibility and 3xGly cleavage by *P. aeruginosa* isolates cultured from the sputum of CF patients, with growth inhibition zones in the 3xGly cleavage-positive isolates being significant larger compared to zones of 3xGly cleavage-negative isolates. Until recently it was stated that AZM is unable to eradicate *P. aeruginosa* by bacterial killing [13]. A recent publication by Buyck et al. however, revealed that growth inhibition of *P. aeruginosa* by AZM depends on the medium used [4]. *P. aeruginosa* strains grown in Mueller Hinton medium have a lower outer membrane permeability compared to strains cultured in for example RPMI. This

results in an increase in susceptibility towards AZM and thus might explain the presence of AZM induced growth inhibition zones we observed on TSA agar plates.

In conclusion, we designed and evaluated a novel 3xGly FRET-peptide substrate for the assessment of virulence in *P. aeruginosa*. Cleavage of the 3xGly FRET-substrate was significantly associated with culture supernatant protease activity, staphylolytic activity, pyocyanin production and the expression of *lasI* in the *P. aeruginosa* QS system. This publication represents the first step in the development of a simple test to determine and monitor *P. aeruginosa* virulence in clinical samples, including the prediction of the effectiveness of QS-inhibiting antibiotic treatments. Preliminary evaluation experiments suggest that this methodology may achieve its greatest potential when monitoring CF patients colonized by, and undergoing treatment for, *P. aeruginosa* infections.

Acknowledgements

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Supplemental data

Table S1. Antibiotic susceptibility of the 97 *P. aeruginosa* strains used in this study [a]

Sample	3xGly cleavage	Specimen	MER	IMP	CAZ	TOB	AMI	CIP	NOR	PIP	TAZ	ATM	COT	COL
B10	+	Blood	R	R	S	R	R	R	R	n/t	n/t	n/t	n/t	n/t
B11	+	Blood	S	S	S	S	S	R	R	I	I	n/t	n/t	S
B12	+	Blood	R	R	R	R	R	R	R	n/t	n/t	n/t	n/t	n/t
B13	+	Blood	R	R	R	R	I	R	R	R	R	R	n/t	S
S12	+	Sputum	n/t	I	S	S	S	S	S	S	S	S	R	S
S13	+	Sputum	S	S	S	S	S	S	S	S	S	n/t	R	n/t
S14	+	Sputum	1	2	R	I	n/t	S	S	n/t	R	n/t	n/t	S
S15	+	Sputum	R	R	S	S	n/t	S	S	I	I	n/t	R	S
S16	+	Sputum	S	S	S	S	S	S	S	S	S	n/t	R	n/t
S17	+	Sputum	2	R	R	R	R	R	R	R	R	R	n/t	S
S18	+	Sputum	2	R	R	R	I	R	R	R	R	n/t	n/t	S
S19	+	Sputum	n/t	S	S	S	S	S	S	S	S	n/t	R	n/t
S20	+	Sputum	S	S	S	S	S	I	R	S	S	n/t	R	n/t
S21	+	Sputum	S	S	S	S	S	S	S	S	S	n/t	R	n/t
S48	+	Sputum (CF)	n/t	S	S	S	R	S	S	S	S	S	S	S
S49	+	Sputum (CF)	n/t	S	S	S	R	S	S	S	S	S	R	S
S50	+	Sputum (CF)	S	S	S	S	R	S	S	S	S	n/t	R	S
S51	+	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S52	+	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S53	+	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S54	+	Sputum (CF)	n/t	S	S	S	R	S	S	S	S	S	R	S
S55	+	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S56	+	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
W15	+	Wound	R	R	R	R	n/t	R	R	n/t	R	n/t	R	S
W16	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W17	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W18	+	Wound	R	R	R	R	n/t	R	R	n/t	R	n/t	n/t	I
W19	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W20	+	Wound	R	R	R	S	n/t	S	S	n/t	R	n/t	R	S
W21	+	Wound	R	R	R	R	I	R	R	R	R	n/t	n/t	2
W22	+	Wound	I	R	R	R	n/t	R	R	n/t	n/t	n/t	n/t	2
W23	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W24	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W25	+	Wound	R	R	S	S	S	R	R	n/t	n/t	n/t	n/t	n/t
W26	+	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W27	+	Wound	I	R	R	R	R	R	R	n/t	R	n/t	n/t	S
W28	+	Wound	S	S	R	S	n/t	S	S	n/t	R	n/t	R	S
B1	-	Blood	S	S	S	R	S	R	R	n/t	n/t	n/t	n/t	n/t
B2	-	Blood	R	R	R	R	n/t	R	R	R	R	n/t	n/t	S
B3	-	Blood	S	S	S	S	S	S	S	n/t	n/t	n/t	n/t	n/t
B4	-	Blood	S	S	S	S	S	S	S	n/t	n/t	n/t	n/t	n/t
B5	-	Blood	n/t	S	I	S	S	S	S	S	n/t	S	n/t	n/t
B6	-	Blood	S	S	S	S	S	S	S	n/t	n/t	n/t	n/t	n/t
B7	-	Blood	S	S	S	S	S	S	S	I	I	n/t	n/t	S
B8	-	Blood	S	R	R	R	I	R	R	n/t	n/t	n/t	n/t	n/t
B9	-	Blood	S	S	S	S	S	S	S	I	I	n/t	n/t	S
S1	-	Sputum	R	R	I	R	n/t	R	R	n/t	R	n/t	n/t	R

S2	-	Sputum	n/t	S	S	S	S	S	S	S	S	n/t	R	n/t
S3	-	Sputum	I	R	R	R	n/t	R	R	n/t	R	n/t	n/t	S
S4	-	Sputum	n/t	R	R	R	R	I	R	I	I	n/t	R	n/t
S5	-	Sputum	n/t	S	R	R	I	R	n/t	R	I	R	R	R
S6	-	Sputum	I	R	R	R	n/t	R	R	n/t	R	n/t	n/t	S
S7	-	Sputum	n/t	R	S	S	S	S	S	S	S	n/t	R	n/t
S8	-	Sputum	R	R	R	I	n/t	S	S	n/t	n/t	n/t	n/t	S
S9	-	Sputum	n/t	S	S	S	S	S	S	S	S	n/t	R	n/t
S10	-	Sputum	4	R	R	I	R	I	S	R	R	n/t	n/t	S
S11	-	Sputum	n/t	S	S	S	S	S	S	S	S	n/t	R	n/t
S22	-	Sputum (CF)	S	S	S	S	R	S	S	S	S	n/t	S	S
S23	-	Sputum (CF)	S	S	S	S	R	S	S	S	S	S	R	S
S24	-	Sputum (CF)	n/t	R	R	R	R	S	S	R	R	R	R	S
S25	-	Sputum (CF)	n/t	S	S	S	S	R	R	S	S	S	R	S
S26	-	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S27	-	Sputum (CF)	n/t	S	S	R	R	R	R	S	S	S	R	S
S28	-	Sputum (CF)	S	S	S	S	R	R	R	S	S	n/t	R	S
S29	-	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S30	-	Sputum (CF)	n/t	R	S	S	I	S	S	S	S	S	R	S
S31	-	Sputum (CF)	R	S	R	R	R	S	I	S	S	R	R	R
S32	-	Sputum (CF)	R	R	S	S	S	R	R	S	S	R	R	S
S33	-	Sputum (CF)	n/t	S	R	R	R	R	R	R	R	R	R	S
S34	-	Sputum (CF)	n/t	S	S	I	R	S	R	S	S	S	R	S
S35	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S36	-	Sputum (CF)	S	R	S	S	S	I	R	S	S	S	R	S
S37	-	Sputum (CF)	n/t	S	S	S	S	S	S	S	S	S	R	S
S38	-	Sputum (CF)	n/t	S	S	R	R	I	R	S	S	S	S	S
S39	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S40	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S41	-	Sputum (CF)	S	R	S	S	S	I	R	S	S	S	R	S
S42	-	Sputum (CF)	R	R	R	S	I	S	S	R	R	R	R	R
S43	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S44	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	n/t	R	S
S45	-	Sputum (CF)	R	R	I	S	n/t	S	S	n/t	I	n/t	R	S
S46	-	Sputum (CF)	S	S	S	S	S	S	S	S	S	S	R	S
S47	-	Sputum (CF)	n/t	R	R	R	R	R	R	R	R	R	R	S
W1	-	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W2	-	Wound	S	S	S	S	S	S	S	n/t	n/t	n/t	n/t	n/t
W3	-	Wound	R	R	8	R	n/t	R	R	n/t	16	n/t	n/t	S
W4	-	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W5	-	Wound	R	R	R	R	n/t	R	R	n/t	n/t	n/t	n/t	S
W6	-	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W7	-	Wound	I	R	R	R	S	I	S	S	R	n/t	n/t	n/t
W8	-	Wound	R	R	R	R	n/t	R	R	n/t	R	n/t	n/t	S
W9	-	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W10	-	Wound	I	I	R	R	S	I	S	I	R	n/t	n/t	S
W11	-	Wound	R	R	R	R	n/t	R	R	n/t	R	n/t	n/t	S
W12	-	Wound	S	S	I	I	n/t	I	I	n/t	R	n/t	n/t	S
W13	-	Wound	S	S	S	S	n/t	S	S	n/t	S	n/t	R	S
W14	-	Wound	S	S	R	R	n/t	R	R	n/t	n/t	n/t	n/t	S

[a] MER: meropenem, IMP: imipenem, CAZ: ceftazidime, AMI: amikacin, CIP: ciprofloxacin, NOR: norfloxacin, PIP: piperacillin, TAZ: piptazobactam, ATM: aztreonam, COT: cotrimoxazol, COL: colistine

Chapter 7

The papain inhibitor (SPI) of *Streptomyces mobaraensis* inhibits bacterial cysteine proteases and is an antagonist of bacterial growth

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Abstract

A novel papain inhibitory protein (SPI) from *Streptomyces mobaraensis* was studied to measure its inhibitory effect on bacterial cysteine protease activity (*Staphylococcus aureus* SspB) and culture supernatants (*Porphyromonas gingivalis*, *Bacillus anthracis*). Further, growth of *Bacillus anthracis*, *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Vibrio cholerae* was completely inhibited by 10 μM SPI. At this concentration of SPI, no cytotoxicity was observed. We conclude that SPI inhibits bacterial virulence factors and has the potential to become a novel therapeutic treatment against a range of unrelated pathogenic bacteria.

Introduction

Papain (EC 3.4.22.2) belongs to clan A of cysteine protease enzymes and is the eponym for the C1 family of proteases which comprises a large number of endopeptidases, although fewer exopeptidases. All members of the papain family contain cysteine and histidine at their active site, forming a catalytic dyad. Papain and related cysteine proteases are widely distributed in the plant kingdom and are believed to act as virulence/defence factors for both hosts and pathogens [1]. Cysteine proteases are also found in bacteria and are known to be virulence factors involved in bacterial pathogenicity [2]. Further, papain-like hydrolases are involved in peptidoglycan turnover in both Gram-negative and Gram-positive bacteria [3, 4], with disruption of amide-hydrolysing autolysins in *Bacillus subtilis,* leading to defective cell wall division and thereby affecting bacterial viability [5]. More recently, the growing resistance of microorganisms to conventionally used antibiotics has meant that cysteine proteases have attracted attention as possible targets for antimicrobial therapy [6]. Indeed, several cysteine proteases have been identified as potential targets for such therapy, including the papain-like staphopains A and B from *Staphylococcus aureus* [7], streptopain (exotoxin B) from *Streptococcus pyogenes* [3], the gingipains RGP and KGP from *Porphyromonas gingivalis* [2], PrtH (FDF) from *Tannerella forsythia* [8] and YopT from *Yersinia enterocolitica* [9].

Recently, we described a novel, heat-resistant protein from *Streptomyces mobaraensis* (SPI) that inhibited the activity of the cysteine protease papain, as well as (to a lesser extent), the activities of cysteine protease bromelain and the serine protease trypsin in the nanomolar range [10]. In the present report, we describe investigations into the inhibitory activity of SPI on bacterial cysteine proteases, i.e. potential virulence factors, and on the growth capability of a range of bacterial pathogens. Our results indicate that SPI has the ability to inhibit secreted bacterial cysteine proteases, as well as bacterial growth, and represent a first step in confirming SPI as a potential broad-spectrum anti-bacterial agent.

Materials and methods

Streptomyces papain inhibitor (SPI)

Preparation of highly purified SPI was performed as described previously [10]. Briefly, *Streptomyces mobaraensis* DSM 40847^T (IPCR 16-22) was cultured at 42 °C for 30 h. The heated cell-free supernatant (70 °C, 30 min) was separated consecutively by

Fractogel EMD TMAE (adsorption at pH 9.0, elution at pH 6.0) followed by two Fractogel EMD SO_3^-- chromatographies, both performed at pH 4.0.

Protease FRET assay

A specialized fluorescent resonance energy transfer (FRET) reporter assay was used to determine the effect of SPI on bacterial proteolytic activity, as previously described by Kaman et al. [11]. Basically, the assay uses a short D-amino acid peptide as a substrate for proteolytic enzymes. This short peptide is linked to both fluorescent reporter and quencher molecules, such that cleavage of the short peptide substrate by specific proteases will result in the spatial separation of reporter and quencher molecules, with a concomitant increase in the fluorescent signal upon excitation of the peptide. For measurement of proteolytic activity, SPI concentrations varying between 10 and 1,000 nM were used and tested in the presence of 16 μM FRET substrate. The peptide sequence of the FRET substrate used varied per organism and was based on data present in previously published articles (Table 1). For analysis, purified enzymes, or 0.2 μm filtered bacterial culture supernatants, were incubated with SPI and FRET substrate at 37 °C for 60 min in a total volume of 50 μl. Residual proteolytic activity was determined in 2 min time intervals during 60 min of incubation at 37 °C. Fluorescence intensity was continuously measured at 530 nm (excitation wavelength of 485 nm) using a FLUOstar Galaxy spectrometer (BMG laboratories, Offenburg, Germany). Negative controls included reaction mixtures prepared in the absence of SPI or brain heart infusion (BHI) medium. After each measurement the relative fluorescence (RF) per minute (RF/min) was calculated, and the RF/min value of the sample without SPI (control) was corrected to 100%.

Inhibition of cysteine protease activity by SPI

The ability of SPI to inhibit cysteine protease activity was tested using purified enzyme staphopain B (SspB; BioCentrum, Krakow, Poland), which is a member of the papain clan

Table 1. FRET-based peptide substrates used in this study

	Micro organism	Protease	Sequence
Purified enzyme	*Staphylococcus aureus*	Staphopain B [a]	FITC-Phe-Arg-KDbc
	Clostridium histolyticum	Collagenase [b]	FITC-Ala-Ala-Gly-Pro-Ala-Ala-KDbc
Culture supernatant	*Bacillus anthracis*	Unknown [a]	FITC-Leu-D-Leu-KDbc
	Porphyromonas gingivalis	Gingipain K/R [a]	FITC-Arg-D-Arg-KDbc

[a] Cysteine protease; [b] Metalloprotease

CA cysteine proteases. A purified metalloprotease, collagenase derived from *Clostridium histolyticum* (Sigma, Zwijndrecht, The Netherlands), was used as a non-cysteine protease control. The enzymes were used in the FRET assay at a final concentration of 60 µg/ml and 80 µg/ml in phosphate-buffered saline (PBS), respectively, in the presence or absence of SPI (1,000 nM). All experiments were performed in triplicate.

Inhibition of bacterial culture supernatant protease activity by SPI

The inhibitory effect of SPI was tested on 0.2 µM filtered culture supernatants of *Porphyromonas gingivalis* and *Bacillus anthracis* after overnight culture in BHI. The supernatants were prepared as described by Kaman et al. [11]. All experiments were performed in triplicate.

Inhibition of bacterial growth

The antimicrobial activity of SPI was evaluated against *Bacillus anthracis* Vollum strain (ATCC 14578), *Staphylococcus aureus* USA300, *Vibrio cholerae* serotype O1 (ATCC 14035) and *Pseudomonas aeruginosa* PAO1 (ATCC 15692). All bacteria were grown overnight in 5 ml BHI medium at 35 °C. The following day, bacteria were cultured to early-logarithmic phase by transferring 100 µl of the overnight culture into 5 ml BHI medium, followed by incubation for 2 h at 35 °C with shaking at 200 rpm. Subsequently, 100 µl of the early-logarithmic culture (1:50 in BHI) was incubated with 100 µl serially diluted SPI (in BHI) or acetate buffer (pH 4.0) at 35 °C and with shaking at 200 rpm. The growth rate was then measured spectrophotometrically at 600 nm at 30 min time intervals for 12 h using the BioScreen apparatus (Thermo Fisher Scientific, Breda, The Netherlands). After incubation, the samples were plated onto Columbia blood agar plates to determine whether the effect of SPI on bacterial growth was bacteriostatic or bacteriocidal. Experiments were performed in triplicate at the biosafety level 3 facility of TNO Defence, Security and Safety (Rijswijk, The Netherlands).

Cytotoxicity assay

The cytotoxicity of SPI on RAW264.7 cells was evaluated in a lactate dehydrogenase (LDH) assay (Roche, Almere, The Netherlands). Cells were incubated for 2 h with culture medium (low control [LC]), 1% Triton-X100 (high control [HC]), SPI or acetate buffer at 37 °C and 5% CO_2. After the incubation, 100 µl aliquots of the supernatants were taken and used in the LDH assay according to the manufacturer's description.

Results

Inhibition of cysteine protease activity by SPI

A concentration of 1,000 nM SPI was sufficient to inhibit the proteolytic activity of purified cysteine protease SspB by approximately 50% (Figure 1). In contrast, the metalloprotease control enzyme, collagenase from *C. histolyticum*, remained active at approximately 95% of the level of activity of the no-SPI control. Interestingly, SPI had a significant inhibitory effect on both types of enzyme activity, though inhibition of SspB reached a much greater significance.

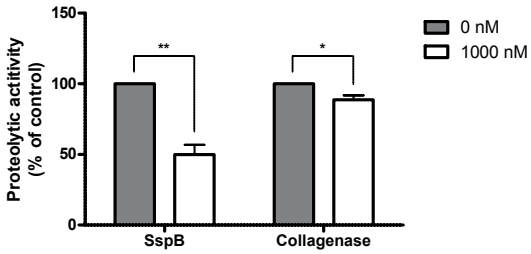

Figure 1. Influence of the papain inhibitor SPI from *Streptomyces mobaraensis* on the proteolytic activity of purified SspB (*S. aureus*) and collagenase (*C. histolyticum*) enzymes. Sixteen micromolar peptide substrate was incubated with 60 µg/ml SspB or 80 µg/ml collagenase in the absence (grey columns) or presence of 1000 nM SPI (white columns). Fluorescence was measured at 37 °C for 60 min. Error bars represent the standard error of the mean (n=3) (** $P < 0.01$; * $P < 0.05$).

Inhibition of bacterial culture supernatant protease activity by SPI

Observations on the inhibitory effect of SPI on *P. gingivalis* and *B. anthracis* overnight culture supernatants indicated that a significant decrease in supernatant protease activity occurred (P <0.05) when we used an SPI concentration of 800 nM in culture supernatants. However, this effect was much more pronounced for *P. gingivalis* than *B. anthracis*, with an SPI concentration of 10 nM achieving a significant inhibition of protease activity. The mean residual protease activity of *P. gingivalis* and *B. anthracis* substrates after 1 h of incubation with 1,000 nM SPI was 3% and 45%, respectively (Figure 2). The 50% inhibitory concentration (IC_{50}) for *P. gingivalis* supernatant protease was 40 nM SPI, and 1,000 nM SPI for *B. anthracis*. Additionally, a dose-response curve was observed in both sets of experiments.

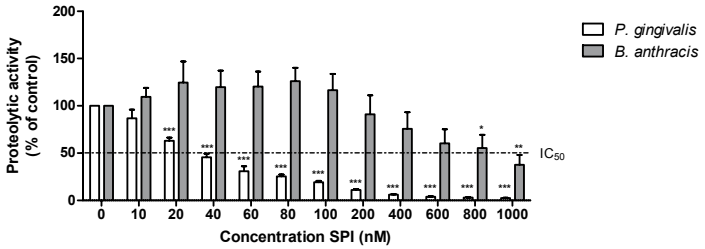

Figure 2. Influence of the papain inhibitor SPI from *Streptomyces mobaraensis* on the proteolytic activity of *P. gingivalis* and *B. anthracis* culture supernatants.Sixteen micromolar peptide substrate was incubated with 40.2 µl culture supernatant and varying SPI concentrations. Fluorescence was measured at 37 °C for 60 min. Error bars represent the standard error of the mean (n=3) (*** P < 0.001; ** P < 0.01; * P < 0.05).

Inhibition of microbial growth

Figure 3 demonstrates that the addition of 10 µM SPI to bacterial cells in the early-logarithmic growth phase was able to completely inhibit the growth of *B. anthracis*, *S. aureus*, *V. cholerae* and *P. aeruginosa.* Further, the growth of *B. anthracis*, *S. aureus* and *V. cholerae* was also delayed and reduced at a concentration of 5 µM SPI. For

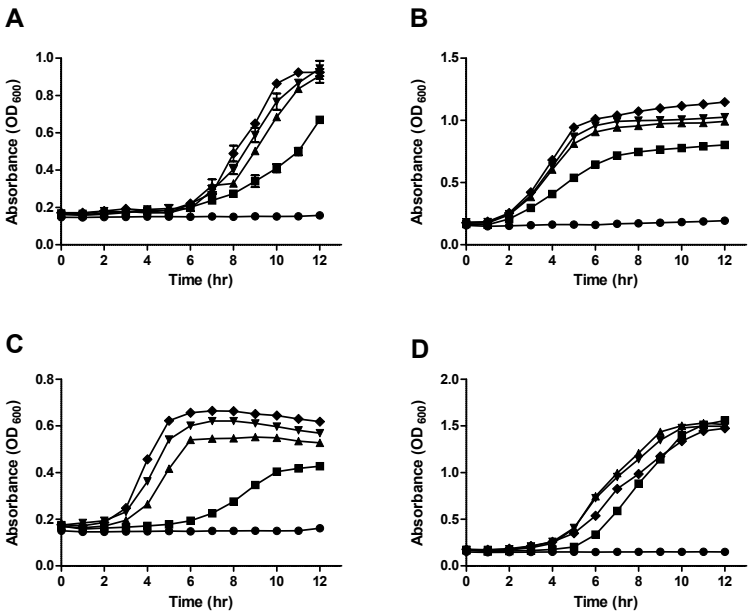

Figure 3. Inhibition of bacterial growth by the papain inhibitor SPI. Growth of *Bacillus anthracis* (A), *Staphylococcus aureus* (B), *Pseudomonas aeruginosa* (C), and *Vibrio cholerae* (D) was inhibited by SPI. Bacteria were cultured at 35°C in the presence of 0 µM (♦), 1.25 µM (▾), 2.5 µM (▴), 5 µM (■), and 10 µM SPI (●). Error bars represent the standard error of the mean (n=3).
3 Tabellen.doc

all organisms, a dose-dependent effect was observed between SPI concentration and inhibition of bacterial growth, although this effect was not linear in scale. Culture of the bacteria surviving the 10 μM SPI experiments on Columbia blood agar generated visible growth for *B. anthracis*, *S. aureus* and *P. aeruginosa* after 72 h of incubation, although no growth was observed for *V. cholerae.* These results indicate that SPI may generate either a bacteriostatic or a bacteriocidal effect on bacterial growth, dependent on the bacterial species.

Cytotoxicity of SPI

SPI was evaluated for its toxicity to RAW264.7 cells at similar concentrations as used in the growth assays. The concentrations at which inhibition of bacterial growth was observed, 10 μM, appeared to be noncytotoxic. Also, the buffer in which SPI was diluted showed no cytotoxicity (all data not shown).

Discussion

During submerged culture, the bacterium *Streptomyces mobaraensis* secretes inhibitory proteins that are active against a variety of endoproteases, including the ubiquitous cysteine protease papain [10]. The isolation of one of these endoprotease inhibitors, the *Streptomyces* papain inhibitor, or SPI, allowed experiments to be performed to investigate the effect of this cysteine protease inhibitor on the proteolytic activity and growth of clinically relevant pathogenic bacteria.

Using internally quenched FRET peptide substrates, and competitive conditions, SPI inhibited the proteolytic activity of purified enzyme, as well as several cell culture supernatants, most likely via the inhibition of important bacterial cysteine protease enzymes. However, these results presume that SPI preferentially inhibits the activity of cysteine proteases. Interestingly, however, genes encoding papain-like cysteine proteases are actually absent in *B. anthracis* [12], although it is known that *B. anthracis* produces NlpC/p60 cell wall hydrolases, which are related to cysteine proteases [5, 13]. These cell wall hydrolases could also be a target for SPI-mediated protease inhibition. Further, our results indicate that SPI has an inhibitory effect on several types of bacterial proteases, including metalloproteases. However, the efficiency of inhibition may vary, dependent on the type of protease being inhibited.

The addition of SPI to growing bacterial cells resulted in a dose-dependent reduction in bacterial growth, a process which may be facilitated by inhibition of cell wall hydro-

lases. These enzymes are part of the cysteine peptidase family and play an important role in bacterial growth, particularly due to their association with cell wall turnover and recycling [4, 14]. For example, papain-related amidases are involved in cell wall degradation [13], while cell wall construction also requires the action of serine proteases, so-called DD-peptidases [15]. This could be one mechanism by which SPI inhibits bacterial growth, although additional experiments are required in order to confirm or deny this hypothesis. In any case, our results indicate that the cysteine protease inhibitor SPI is a good candidate for further research, particularly with respect to its mode of action and the range of bacterial species it inhibited. From our own experiments, SPI appears to be effective as an inhibitory agent against a wide range of important and clinically relevant bacterial species, including species comprising Gram-positive cocci, Gram-positive rods and Gram-negative rod phenotypes, without being cytotoxic. The work suggests that SPI may have the potential to become a novel broad-spectrum antimicrobial agent for the treatment of clinically relevant infectious diseases. Additionally, SPI is a glutamine and a lysine donor protein of transglutaminases [10], a property that could be exploited to facilitate the preparation of SPI-containing antimicrobial protein sponges and foils for use in medical applications.

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General discussion

Chapter 8

Summarising discussion

Proteases are essential for bacterial growth and proliferation, and contribute to bacterial virulence. Their secretion into the micro-environment, as well as their importance in bacterial viability and pathogenicity, makes them suitable targets for diagnostic and therapeutic purposes. For example, a "point-of-care" test based on the detection of bacteria-related proteolytic activity, the BANA-test, is commonly used to diagnose periodontal disease [1]. Additionally, the usefulness of proteases as therapeutic targets has already been demonstrated in viral infections, e.g. in the treatment of hepatitis C and HIV-related infections; such strategies rely on the inhibition of viral proteases as a mechanism of action, and a variety of drugs have been developed to target viral proteases [2-4]. The data presented in this thesis indicates that bacterial proteases also represent potential targets for novel diagnostic techniques and therapeutic strategies with potential applications in the clinical microbiology laboratory.

Specific detection of bacterial proteolytic activity

The detection of bacterial proteolytic activity in clinical specimens is challenging due to the presence of eukaryotic proteases in biological samples. For example, human proteases may cross-react and cleave a substrate employed with the aim of specifically detecting bacterial proteases. However, it has been shown that, for example, the non-specific cleavage of antimicrobial peptides by human proteases could be significantly reduced by exploiting the chiral nature of amino acids via the addition of D-amino acids to L-amino acid peptide sequences [5]. The presence of D-amino acids is uncommon in eukaryotic proteins; D-amino acids are only detected in the brain. However, the blood-brain barrier prevents the transport of the D-amino acid-specific proteases present in the brain to the circulation and/or to the other bodily fluids or organs which are accessible and suitable for sampling and subsequent testing in the laboratory [6]. Importantly, D-amino acids are abundant in bacterial peptidoglycan (PGN). During bacterial growth, both disassembly and assembly of the cell wall occurs, requiring the production of proteases that recognize and cleave the D-amino acid bonds present in the PGN stem peptide [7]. Therefore, D-amino-acid-containing substrates could hypothetically be used to specifically detect bacterial proteolytic activity. In addition to the presence of D-amino acids, the length of the substrates, i.e. the number of amino acids present, is also an important factor which affects non-specific cleavage by eukaryotic proteases. A large protease substrate contains more potential non-specific cleavage sites than a small protease substrate. Essentially, for diagnostic purposes, care has to be taken to limit the

number of false positives, which can be realised by using short D-amino-acid-containing peptide substrates for the detection of bacteria.

With this in mind, a FRET-peptide substrate library comprising 115 short, L- and D-amino-acid-containing peptides was designed. This library was used to evaluate the feasibility of these substrates to specifically detect bacterial protease cleavage activity (**Chapter 2**). Firstly, the bacterial specificity of the D-amino acid substrates was examined using purified and commercially available enzymes of both eukaryotic and prokaryotic origin. Using this limited set of enzymes, it was observed that only bacterial proteases cleaved the D-amino-acid-containing substrates. More specifically, an interesting finding was that among the bacterial proteases tested, only enzymes involved in either nutrition or housekeeping degraded the D-amino-acid-containing substrates. In contrast, bacterial proteases associated with virulence tended to recognise and degrade substrates that predominantly consisted of L-amino acids. This finding can be explained by the fact that bacterial virulence factors (such as collagenase from *Clostridium* spp.) are known to target eukaryotic proteins, and that these proteins predominantly consist of L-orientated amino acids [8,9]. Subsequent experiments showed that substitution of an L-amino acid for its D-enantiomer abolished the degradation of FRET-peptide substrates by the human proteases present in serum (**Chapter 3 and 4**). This was not the case when saliva obtained from healthy volunteers was used, as cleavage of D-amino-acid-containing peptides was observed. Using human saliva, a lower extent of D-amino-acid-containing peptide cleavage was observed compared to the cleavage of all-L-amino acid peptide substrates. This finding is most likely due to the fact that saliva is loaded with many different types of bacterial species, even in the case of a healthy oral state [10]. Overall, the conclusions from the studies presented in **Chapters 2, 3 and 5** indicate that the use of short D-amino acid FRET-peptide substrates in proteolytic detection assays increases the specificity of substrate cleavage by bacterial proteases.

D-amino acid peptide substrates for the diagnosis of bacterial infections

Despite its time consuming and laborious nature, culture-based diagnosis remains the most reliable and frequently used technique in the routine diagnostic clinical microbiology (Table 1). In order to decrease the time to diagnosis, various sophisticated nucleic acid- and antibody-based alternatives have been developed and utilised [11]. Compared

to culture, these methods are more rapid, but do not provide information on the viability of the microorganism detected. One of the most promising new techniques for microbial diagnosis combines the culture-based detection of living microorganisms with rapid identification by mass spectrometry (MS) [12,13]. MS is used as an extension of conventional culture and allows the microbiologist to rapidly identify the cultured microorganism by comparison of MS patterns with a spectral reference database. A disadvantage of this system is that the purchase of a mass spectrometer is costly, which might make this method less attractive to smaller diagnostic laboratories [14]. Currently, the direct analysis of clinical specimens using MS remains difficult. Therefore, a need still exists for alternative rapid, simple and inexpensive methods to detect and identify bacterial pathogens directly in clinical specimens.

The secretion of bacterial proteases into the micro-environment makes them accessible as targets for the detection of microorganisms in clinical samples. In addition, the detection of proteolytic activity with the use of fluorogenic protease-based substrates could provide a rapid, simple and generally inexpensive diagnostic technology for the detection of bacterial pathogens. However, as previously mentioned, a major drawback of this technique is that most substrates cross-react with unrelated, non-microbial proteases, requiring time-consuming pre-isolation of the protease(s) of interest [15,16]. As a consequence, the literature available on the applicability of protease-based microbial detection is scarce [17-19]. Wildeboer et al. described the diagnosis of *Pseudomonas aeruginosa*-related wound infections via the detection of proteolytic activity [20]. Although the proteolytic activity of wound fluid samples correlated with the presence of *P. aeruginosa,* the authors could not exclude the involvement of host protease activity. Thus, it is not clear whether the proteolytic activity which the authors detected was actually due to proteases secreted by *P. aeruginosa* or alternatively, was the result of enzymes produced by the host in response to the presence of the microorganism [21].

Table 1. Advantages and disadvantages of a selection of methods to diagnose infectious disease.

	Advantages	**Disadvantages**
Conventional culture	Reliable, provides information on viability	Laborious, time-consuming
Nucleic-acid-based diagnosis	Rapid, high sensitivity, high specificity	Expensive, laborious, no information on viability
Antibody-based diagnosis	High specificity, high sensitivity	Expensive, laborious, no information on viability
Mass spectrometry (i.c. with culture) [a]	Relatively rapid, provides information on viability	Requires acquisition of expensive equipment
D**-amino acid FRET-technology**	Rapid, simple, provides information on viability	Low sensitivity

[a] i.c.: in combination

Porphyromonas gingivalis

Periodontitis is an inflammation of the periodontium induced by microorganisms that adhere to and grow at the gingival margin [22]. Measuring pocket depth, in combination with culture and quantitative PCR, is currently the most commonly used strategy to diagnose periodontitis [23]. *Porphyromonas gingivalis* is often implicated in infections of the periodontium. This microorganism is known to secrete highly proteolytic active virulence factors, the "gingipains" [24], which makes *P. gingivalis* well suited for the development of a specific protease-based detection technology. In this respect, screening of a D-amino acid FRET-peptide substrate library with culture supernatants of *P. gingivalis* revealed five *P. gingivalis*-specific substrates (**Chapters 2 and 3**). Experiments using gingipain knock-out strains revealed that the proteolytic activity detected in *P. gingivalis* culture supernatants was gingipain-specific. Furthermore, the specificity of these FRET-peptide substrates was evaluated using crevicular fluid, a fluid from the gingival crevice. It was found that the peptide substrates were able to reliably detect *P. gingivalis* in patient-derived clinical material. In a comparative study, the FRET-peptide substrates and BANA, a commercially available test for the protease-based diagnosis of periodontal disease, were evaluated [25]. This rapid "point-of-care" periodontal test is approved for use in routine dental practice and utilises a substrate consisting of a single L-orientated arginine residue (BANA test strips; www.oratec.net). We found that the sensitivity and specificity of the FRET-peptide substrates for the detection of *P. gingivalis* - of up to 60% and 100%, respectively - were higher than that of the commercial BANA test, which scored 40% and 85%, respectively. Additionally, in **Chapter 5**, the sensitivity and specificity of the *P. gingivalis* specific D-amino acid substrates were compared to two other diagnostic methods: quantitative PCR (qPCR) and culture. Subgingival plaque and salivary samples derived from patients with and without peri-implant infections were examined for the prevalence of *P. gingivalis* using the above-mentioned techniques. When culture was used as the "gold standard", the FRET technique showed a sensitivity and specificity for the detection of *P. gingivalis* of 67% and 100%, respectively, in subgingival plaque material. Using saliva samples, the sensitivity and specificity values of the FRET technique were 63% and 75%, respectively. For both sample types, the sensitivity of the qPCR-based test was higher than that of the FRET-based technique, although the specificity of qPCR was found to be lower. In summary, these results suggest that the novel D-amino acid FRET-based assay might be a valuable addition to the routine laboratory methods currently used for the detection of *P. gingivalis* in saliva.

Bacillus anthracis

Biological warfare agents (BWA), such as *Bacillus anthracis* and *Clostridium botulinum,* are well-known for their high pathogenicity. Most BWA infections generate non-specific clinical symptoms, making diagnosis relatively difficult [26]. Furthermore, the antibiotic therapies commonly used to treat BWA pathogens, e.g. doxycycline and penicillin, are only thought be effective if administered within 24 h post-exposure [27]. Therefore rapid detection of BWA pathogens is critical and will enhance the success and outcome of antibiotic therapy [28]. In our view, FRET-technology could be valuable for the rapid diagnosis of BWA-related infections. In this respect, FRET-peptide substrates for *B. anthracis* were designed and exposed to a broad spectrum of bacterial culture supernatants, including two Category A BWA: *C. botulinum* and *Yersinia pestis* [29]. It appeared that the *B. anthracis* D-amino-acid-containing substrates, specifically BikKam1 (Leu-D-Leu), were specific for *B. anthracis* and other closely related *Bacillus* spp. (**Chapters 2 and 5**).

To further explore the ability of the FRET-peptide substrate assay to diagnose an anthrax infection, the sera of *B. anthracis*-infected mice were analysed using the BikKam1 substrate. At 48 h post-infection, when the infection was systemic and the mice were clearly affected by the disease, BikKam1 degradation products could be detected in the sera of infected mice by MS analysis. This indicates that the FRET-peptide substrate assay technology might be applicable to adaptation for the detection of *B. anthracis* in humans. Based on the promising results described above, it is suggested that the BikKam1 substrate might also be useful in the detection of so called "anthrax letters", i.e. letters containing *B. anthracis* spores. Next, a methodology to detect *B. anthracis* spores within 4 h using the BikKam1 peptide substrate was successfully developed. One of the methods currently used by the U.S. Postal Offices to identify "anthrax letters" is the PCR-based technique marketed by Cepheid (www.cepheid.com/tests-and-reagents/anthrax). Using this method, it is possible to detect *B. anthracis* spores within 45 min. However, though the sensitivity of this PCR-based technique was higher than FRET-peptide based detection, the FRET-peptide substrate assay approach presented in **Chapter 4 and 5** is easier to perform, requires a minimal number of experimental steps, and provides information on the viability of the microorganism detected. Additionally, and in contrast to PCR-based methods, FRET-peptide technology can be easily adapted for use in point-of-care devices (or "hand-held devices"). The detection of *B. anthracis* using a FRET-peptide based point-of-care device could be useful for the military in war zones and battlefields where biological weapons might be deployed. Though these weapons

are banned by international regulations and conventions, recent developments in the Middle East have shown that these international treaties apparently do not stop the deployment and use of chemical and/or biological weapons. Therefore, the ability to rapidly detect BWA remains crucial. In this respect, the first attempts to develop the BikKam1 substrate into an electrochemical biosensor for the detection of *B. anthracis* have already been made [30].

Further characterization and identification of the enzyme(s) involved in BikKam1 cleavage will enhance our ability to improve the novel methodology described in this thesis. When the enzyme responsible for cleavage of the BikKam1 peptide substrate is identified, specific stimulators of the assay, such as L-cysteine, could be added to the FRET assay to increase its limit of detection. One candidate enzyme which may be potentially responsible for the cleavage of BikKam1 was recently described by Sela-Abramovich and co-workers, who discovered the cysteine peptidase BA1952, a peptidase present in the sera of anthrax-infected mice [31]. This peptidase is a member of the NlpC/p60 endopeptidase family which hydrolyses the D-γ-glutamyl-meso-diaminopimelate linkage in PGN [32].

Detection of bacterial proteolytic activity to monitor virulence

Bacterial proteases play an important role in virulence via the degradation of host proteins [33,34]. The detection of these (proteolytic) virulence factors could provide the microbiologist with important information on the severity of disease, factors associated with adverse prognosis, and the likely clinical outcome. A good candidate microorganism for the evaluation of such FRET-peptide based virulence monitoring techniques is *Pseudomonas aeruginosa,* as infections with this pathogen are associated with a high morbidity and mortality in the hospital environment, and *P. aeruginosa* is known to secrete large quantities of proteolytic virulence factors [35]. Secretion of a high percentage of these *P. aeruginosa* virulence factors is under the control of the quorum sensing (QS) system, which coordinates the expression and production of bacterial proteins related to population density. The QS-system regulates functions such as biofilm formation, swarming, and the secretion of virulence factors, and thus contributes to bacterial pathogenesis [36]. Therefore, as well as having potential for monitoring virulence, the detection of *P. aeruginosa* proteases might be informative

in terms of examining the effectiveness of QS-inhibitors designed as an alternative to current antimicrobial treatment. Recently, the ability of a *P. aeruginosa* isolate to produce QS-related proteins, the rhamnolipids, was used to predict the therapeutic effect of QS-inhibitors [37]. The production of rhamnolipids is determined by culturing the bacterium overnight on agar plates in the presence of methylene blue and cetyl trimethylammonium bromide [38]. Therefore, a FRET-peptide based detection assay for QS-related proteases is a potentially more rapid method to screen for QS-system activity. In this research, a FRET-peptide substrate for *P. aeuruginosa* was designed and evaluated, and its association with virulence factor production and susceptibility towards a QS-inhibiting antibiotic was evaluated (**Chapter 6**). It was observed that cleavage of the FRET-peptide substrate, G-G-G, was QS-related and associated with the production of two important *P. aeruginosa* virulence factors: pyocyanin and the LasA protease. Moreover, we observed that cleavage of the FRET-peptide substrate correlated with the susceptibility of *P. aeruginosa* isolates to azithromycin, a macrolide antibiotic that inhibits QS in *P. aeruginosa* [39]. These preliminary results suggest that the use of the novel triglycine FRET-peptide substrate could provide useful information on pathogen virulence and the severity of an infection, thereby providing clinicians with potentially useful data regarding the virulence potential of *P. aeruginosa* isolates infecting or colonising individual patients.

Bacterial proteases as therapeutic targets

As well as their potential as diagnostic markers, bacterial proteases may also be considered as targets for the treatment of bacterial infections [40,41]. The effectiveness of such a treatment strategy has already been proven for viral infections. Currently, therapy applied for the treatment of hepatitis C is based on inhibition of the NS3-protease, an enzyme necessary for replication of the hepatitis C virus [42]. This provides a basis for the use of bacterial protease inhibitors to attenuate the progression and severity of bacterial infections. In collaboration with Prof. dr. Fuchsbauer and Dr. Zindel, we examined the applicability of *Streptomyces* Papain Inhibitor (SPI), which is isolated from the bacterium *Streptomyces mobaraensis,* in the treatment of bacterial infections. In a previous study, it was demonstrated that SPI specifically inhibits the activity of cysteine proteases [43]. In addition, it is known that various cysteine proteases of bacterial origin are involved in bacterial virulence. Therefore, the effect of SPI on the bacterial cysteine proteases SspB (*Staphylococcus aureus*) and gingipains (*P. gingivalis*)

was investigated (**Chapter 7**). SPI inhibited the proteolytic activity of these proteases by 50% and 100%, respectively. Interestingly, it was shown that SPI is able to inhibit the bacterial growth of a broad range of bacteria, including *B. anthracis*, *S. aureus*, *P. aeruginosa,* and *Vibrio cholerae,* in a bacteriostatic manner (**Chapter 7**). The ability of cysteine proteases to inhibit bacterial growth was reported for the first time in 1989 by Bjork et al. [44]. A synthetic peptide based on the structure of a human cysteine protease exhibited antibacterial activity against a large number of bacterial strains. The efficacy of cysteine protease inhibitors might be related to the fact that several cysteine proteases, e.g. NlpC/p60 hydrolases, are involved in bacterial cell wall synthesis [7]. These proteases serve as autolysins and are essential for PGN synthesis, cleaving PGN between the D-isoglutamic and mesodiaminopimelic acid residues. Inhibition of these proteases could inhibit bacterial cell wall synthesis, which may possibly explain the observed ability of SPI to attenuate bacterial growth. However, further experiments are required in order to reveal the exact mechanism of SPI-mediated bacterial growth inhibition. Additionally, further studies are required to determine if SPI can be used to reduce or inhibit the virulence of cysteine protease-secreting bacteria such as *S. aureus* and *P. gingivalis*. Based on the results presented in this thesis, it is possible to conclude that SPI is a protease inhibitor with promising anti-bacterial and/or anti-virulence activity which is worthy of further investigation.

Future perspectives

The rapid detection and initiation of appropriate treatment for microbial infections is usually associated with an improved clinical outcome. Although the sensitivity of FRET-peptide substrates is somewhat lower than that of PCR, the D-amino-acid-containing FRET-substrates described in this thesis have the potential to be developed for use as a rapid diagnostic tool for bacteria-related infectious diseases. However, bacterial proteolytic activity, and thus the ability of proteases to cleave a particular peptide substrate, depends on a number of factors including pH, temperature, and the protease stimulators/inhibitors present in the surrounding environment. In addition, the sensitivity of proteolytic substrate-based technologies depends on the variety and concentration of the proteases secreted by bacterial pathogens. This may be affected by the availability of nutrition, the growth phase of the bacterium, and the presence of other organisms in the microenvironment. Therefore, additional studies are needed to further optimise the FRET-peptide substrate-based diagnostic technology. These studies should aim to

identify the optimal activity and secretion conditions for the protease of interest. This knowledge will help to define the suitability of the use of D-amino-acid-containing FRET-peptide substrates under various clinical conditions, and provide information that may be useful in altering the microenvironment of a clinical specimen in order to facilitate increased protease production with the aim of improving the sensitivity of the assay. Finally, the applicability of FRET-peptide substrates and (novel) protease inhibitors for the detection and treatment of antimicrobial resistance associated with bacterial proteases should be explored.

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Chapter 9

Nederlandse samenvatting

De huidige methoden voor het identificeren van micro-organismen in klinische materialen zijn tijdrovend, complex of missen sensitiviteit en specificiteit. Deze methoden zijn gebaseerd op het aantonen van bacterie-specifieke producten in klinisch materiaal, zoals celwandeiwitten, DNA, RNA of metabolieten. Bacteriële proteasen (eiwitafbrekende enzymen), zijn theoretisch geschikte kandidaten voor het identificeren van levende micro-organismen in klinisch materiaal; het aantonen van proteolytische activiteit is namelijk snel en simpel. Proteolytische activiteit kan gemeten worden met behulp van substraten welke gelabeld zijn met fluorescente groepen (FRET-substraten). Deze FRET-substraten bestaan, net als eiwitten, uit aminozuren. Wanneer proteasen de aminozuursequentie van de FRET-substraten herkennen en afbreken kan het fluorescente signaal met een externe lichtbron worden geactiveerd en gedetecteerd. Hoewel aminozuren in twee vormen voorkomen, namelijk als linksdraaiende (L-) en rechtsdraaiende (D-) aminozuren, bestaan eigenlijk alle FRET-substraten beschreven in de literatuur uit L-aminozuren. Gebaseerd op het feit dat van alle levensvormen alleen bacteriën unieke proteasen bezitten die D-aminozuren kunnen afbreken werd er onderzoek verricht naar de ontwikkeling van bacterie-specifieke FRET-substraten; korte substraten waarin tenminste één D-aminozuur aanwezig is. Hypothetisch kunnen deze substraten alleen worden afgebroken door bacteriële proteasen en niet door de proteasen afkomstig van de gastheer die in het klinisch materiaal aanwezig kunnen zijn.

In **Hoofdstuk 2** werd een bibliotheek ontworpen van korte, D-aminozuur bevattende FRET-substraten. Experimenten wezen uit dat alleen de proteasen die een rol spelen in de cellulaire huishouding van bacteriën in staat waren de D-aminozuur bevattende FRET-substraten af te breken. Eukaryotische (gastheer) proteasen en bacteriële proteasen die een rol spelen in virulentie braken alleen de FRET-substraten af waarin geen D-aminozuren aanwezig zijn. Vervolgens werd de toepasbaarheid van de D-aminozuur bevattende FRET-substraten in de diagnostiek van bacteriële infecties onderzocht. Hiertoe werd de substraatbibliotheek (**Hoofdstuk 2**) gescreend met kweek supernatanten afkomstig van een breed bacterieel spectrum. Dit leverde onder andere vijf FRET-substraten op die specifiek waren voor *Porphyromonas gingivalis*, een bacterie welke betrokken is bij tandvleesontstekingen en parodontitis (**Hoofdstuk 3**). De specificiteit en sensitiviteit van deze substraten werd vergeleken met bestaande conventionele kweek methoden en kwantitatieve PCR in subgingivale plaque en speeksel van personen met en zonder *P. gingivalis* gerelateerde parodontitis. Uit deze experimenten bleek dat met behulp van de D-aminozuur bevattende FRET-substraten een hogere specificiteit behaald werd in

vergelijking tot PCR. Echter, de sensitiviteit van de kwantitatieve PCR lag hoger dan die van de D-aminozuur bevattende FRET-substraten (**Hoofdstuk 4**).

Naast substraten voor de detectie van *P. gingivalis* werd er een FRET-substraat (BikKam1) ontworpen die specifiek was voor *Bacillus anthracis*, een bacterie die in verband wordt gebracht met bioterrorisme. Het bleek mogelijk om binnen 4 uur anthrax sporen aan te tonen met behulp van het BikKam1 substraat. Dit geeft aan dat het BikKam1 substraat mogelijk gebruikt zou kunnen worden om "anthrax brieven" te identificeren (**Hoofdstuk 5**). Daarnaast werd de potentie van dit substraat onderzocht om een anthrax infectie te detecteren in muizen geïnfecteerd met *B. anthracis* sporen. Achtenveertig uur na infectie kon met behulp van massa spectroscopie (MS) een BikKam1 afbraakprodukt worden aangetoond in het serum van ernstig zieke muizen (**Hoofdstuk 5**). De resultaten beschreven in de **Hoofdstukken 3-5** laten zien dat D-aminozuur substraten de potentie hebben voor gebruik in de diagnostiek van infectieziekten. Echter additioneel onderzoek met betrekking tot de optimalisatie van proteolytische activiteit is nodig om de toepasbaarheid van D-aminozuur substraten in de diagnostiek te verhogen.

Een aantal bacteriële proteasen zijn in staat relevante eiwitten van de gastheer af te breken en spelen hierdoor een belangrijke rol in virulentie. Detectie van bacteriële protease-activiteit zou dus naast identificatie van het micro-organisme ook informatie kunnen geven over de pathogeniciteit van een bacterie. Omdat *Pseudomonas aeruginosa,* een bacterie betrokken bij infecties van brandwonden en luchtweginfecties, veel verschillende proteolytische virulentie factoren uitscheidt hebben we deze bacterie geselecteerd om de toepassing van FRET-substraten in het voorspellen van bacteriële pathogeniciteit te onderzoeken. Afbraak van het substraat ontworpen voor *P. aeruginosa* bleek inderdaad te correleren aan de secretie van twee quorum sensing (QS) gerelateerde virulentie factoren, het LasA protease en pyocyanine (**Hoofdstuk 6**). Daarnaast lijkt er een verband te zijn tussen degradatie van het *P. aeruginosa*-substraat en de gevoeligheid voor het QS-remmende antibioticum azithromycine. Het onderzoek beschreven in **Hoofdstuk 6** laat dus zien dat FRET-substraten mogelijk gebruikt kunnen worden om de ernst van een infectie in te schatten. Omdat proteasen een rol kunnen spelen in virulentie van een micro-organisme doen verschillende wetenschappers onderzoek naar het therapeutisch effect van proteaseremmers. Het effect van remmers op proteolytische activiteit wordt voornamelijk geanalyseerd met behulp van FRET-substraten. In **Hoofdstuk 7** beschrijven we het effect van een cysteine proteaseremmer geïsoleerd uit de bacterie *Streptomyces mobaraensis* (SPI) op virulentie factoren van *P. gingivalis* (gingipains) en *Staphylococcus aureus* (SspB). In beide gevallen werd de proteolytische

activiteit geremd na toevoeging van SPI. Omdat het bekend is dat er cysteine proteasen betrokken zijn bij de synthese van de bacteriële celwand werd ook het effect van SPI op de groei van de bacteriën *Vibrio cholerae*, *S. aureus*, *B. anthracis* en *P. aeruginosa* onderzocht. Hieruit bleek dat met een lage concentratie SPI de groei van alle vier de bacteriën geremd kon worden. SPI zou dus mogelijk in de toekomst gebruikt kunnen worden als breed spectrum antibioticum in de behandeling van infectieziekten.

Samenvattend, de korte, D-aminozuur bevattende FRET-substraten beschreven in dit proefschrift hebben de potentie om gebruikt te worden in de diagnostiek van bacteriële infectieziekten. Naast de identificatie van bacteriën kunnen FRET-substraten ook toegepast worden in het volgen van het verloop van een bacteriële infectie en het effect van antimicrobiële therapie. Tevens kunnen FRET-peptide substraten bijdragen in onderzoek naar nieuwe antimicrobiële middelen.

Appendices

Dankwoord

De activiteit van een protease is afhankelijk van een aantal omgevingsfactoren waaronder pH, temperatuur en de aanwezigheid van stimulatoren/remmers. Ook het succes van een promotie is sterk afhankelijk van zijn omgeving. De reorganisatie die tijdens mijn promotie plaatsvond bij TNO leidde tot een sterke daling in pH en temperatuur. Echter, door de aanwezigheid van de juiste mensen (stimulatoren) op de juiste momenten is het toch nog gelukt een mooi boekje af te leveren. Deze personen wil ik dan ook graag in het bijzonder bedanken.

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Curriculum vitae

Wendy Esmeralda Kaman was born on February 3rd, 1980 in `s-Gravenhage. After finishing her secondary education (HAVO) at the Zandevelt College in `s-Gravenzande in 1996, she started studying Medical Biology at the Hogeschool Rotterdam. She followed her final internship at the Experimental Hepatology Department of the Academisch Medisch Centrum in Amsterdam. There, she studied the effect of fibrosis on hepatocyte transplantation in patients with progressive familial intrahepatic cholestasis (PFIC3). In November 2001, she graduated and in the same year she started working as a research technician at the Human Genetics Department of the Leids Universitair Medisch Centrum. In the research group of Dr. Judith van Deutekom she worked on therapeutic anti-sense induced exon skipping in Duchenne patients. In 2003 she switched jobs and continued scientific research at the Defence Security and Safety Department of TNO, Rijswijk. At TNO she was involved in research towards the detection, diagnosis and treatment of infections related to biological warfare agents. One of the projects she worked on was based on the diagnosis of biological warfare related infections using FRET-peptide substrates with Dr. Floris Bikker as project leader. Her enthousiasm for the subject of this project resulted in the start of her PhD under supervision of Dr. Floris Bikker (TNO and later ACTA), Dr. John Hays (Erasmus MC) and Prof. dr. dr. Alex van Belkum (Erasmus MC and later bioMérieux) in 2009. In the same year she participated in the 6th TNO SBIR Valorisation Grant program and gained funding for a feasibility study to validate the applicability of FRET-peptide substrates in the diagnosis of hospital acquired infections. In collaboration with ACTA and TMM partners she was awarded a STW Valorisation Grant for the development of a rapid FRET-based diagnostic test for oral pathogens in 2010. On Christmas day 2010, she gave birth to a daughter, and named her after the best season of the year; Lente. In 2010, her PhD project was continued at the Erasmus Medical Center at the Department of Medical Microbiology and Infectious Diseases in Rotterdam. At the Erasmus MC she combined her PhD research with activities for the TEMPOtest-QC KP7 project and the KP7 project, PARCIVAL. Wendy currently continues her research in the Department of Medical Microbiology and Infectious Diseases at the Erasmus MC finishing the PARCIVAL project.

List of publications and patents

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· PhD Day Erasmus MC	2011
· Microbial Pathogenesis Workshop	2011
National and International conferences	
· BioDefence 2009 (oral presentation)	2009
· Scientific Spring Meeting NvMM (poster presentation)	2009
· Scientific Spring Meeting NvMM	2011
· 4th Annual Symposium on Host Defence Peptides (oral presentation)	2011
· MolMed day (poster presentation)	2012
· Scientific Spring Meeting NvMM (poster presentation)	2012
· MolMed day (poster presentation)	2013
· Scientific Spring Meeting NvMM (oral presentation)	2013
Scientific meetings	
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Supervision of students	
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· Supervision of medical students "VO Infectieziekten"	2010–2013

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