Evaluation of Carboxy-terminal processing proteases as antibiotargets in *Pseudomonas aeruginosa*

Dissertation

zur
Erlangung des Doktorgrades Dr. rer. nat.
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vorgelegt von

drs. Henricus Lubbertus Hoge

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The design of the cover and back shows a Dragon curve or Heighway dragon and is a mathematical fractal. A fractal is a mathematical set that exhibits a repeating pattern which is infinitely self-similar. The dragon curve was used by Michael Crichton in his book “Jurassic Park”. This book woke in me the interest for molecular biotechnology. The fluorescent green color of the dragon curve on the cover represents *Pseudomonas aeruginosa*. This bacterium is represented by the fractal as the result of the multiple replications of one bacterium. At a far distance it looks as a unorganized mass of lines/ bacteria. But when you look in more detail and interest to the mass you’ll discover a highly organized and structured system of elegance and beauty which can be highly devastating. This whole system has been build on the basis of one simple repeating principle.
Die vorliegende Arbeit wurde am Zentrum für Quanten Biowissenschaften/Translationaler Peptid Forschung der Universität Ulm angefertigt.

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Evaluation of Carboxy-terminal processing proteases as antibiotargets in *Pseudomonas aeruginosa*

Rien Hoge
Voor Ingeborg, Berndt, Janne en Aniek

mijn ouders

oma van Rijswijk
“Whatever it is you seek, you have to put in the time, the practice, the effort. You must give up a lot to get it. It has to be very important to you. And once you have attained it, it is your power. It can’t be given away: it resides in you. It is literally the result of your discipline.”

Ian Malcolm’

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff
Foreword

The following thesis is the result of the research I have done to obtain a PhD. There are three main reasons that made this research for me very valuable.

First, as teenager I was already interested in medicines. It is remarkable that such a small amount of a substance can have enormous influences on our health and well being. Infectious diseases are one of the major health problems in the world and influences the all day lives of a substantial portion of the world. This research has enabled me to search for new medicines that could treat infectious diseases.

Secondly, besides that I’m intrigued by medicines I also have always been interested in molecular biology. This interest was released after reading Michael Crichton’s book “Jurassic Park” which is by no means the equivalent to the highly successful film. Michael Crichton had the ability to foresee the possibilities of new technologies not merely that of biotechnology. Reading this book gave me the insight in a until then for me unknown toolbox of nature which is very powerful. The research executed in this thesis gave me the possible to use this toolbox in its full depth and to skill me further in its techniques.

Thirdly, this research was part of a European consortium with several partner organizations. I am convinced that collaboration in scientific research is most useful and will increased the success rate. Therefore I have always requested and searched for experts in a particular field or technique. The consortium enabled me to discuss the joint project and science in general with PhD students, Post docs and other researchers from all over Europe and beyond eg. Turkey, Croatia, France, Germany, United Kingdom, Italy, Cyprus, Spain, Austria, Poland and Malaysia. During my research I was able to travel to several places and institutes in the world for meetings and for doing research. The places I have been are: Ulm University, (Ulm, Germany); Forschungszentrum Jülich (Jülich, Germany); Heinrich Heine University (Düsseldorf, Germany); Institut de Microbiologie de la Méditerranée-CNRS (Marseille, France), University of Nottingham (Nottingham, United Kingdom), Imperial College (London, United Kingdom), University of Groningen (Groningen, the Netherlands) and the Radboud University Nijmegen (Nijmegen, the Netherlands). And even beyond Europe. I had the honor to research and reside for several months at the Massachusetts General Hospital, Harvard Medical School in Boston, Massachusetts, USA.

Based on these three arguments I am very grateful to be able to deliver and present the fore lying thesis.

Rien
Koekange, December 2014
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Summary

Since the development and intensive use of antibiotic drugs starting at the beginning of the 20th century also the problem of resistance towards these agents has emerged. As the development and introduction of novel antibiotics have declined over the years the problem of multi-drug resistance is becoming alarming by the day. The mechanism of today’s antibiotics is to destroy or inhibit growth of a pathogenic bacterium which leads to high selection pressure and development of resistance. The aim of this thesis was to identify novel targets for the development of antibiotics that only disarm the pathogen in order to reduce its virulence and by doing so reducing the selection pressure and development of resistance.

*Pseudomonas aeruginosa* was used within this thesis as the model organism. *P.aeruginosa* is an aerobic Gram-negative bacterium and opportunistic pathogen that is an important cause of both community-acquired and hospital-acquired infections. Especially in patients with Cystic Fibrosis *P.aeruginosa* causes high morbidity and mortality.

In this thesis the focus lays on the group of Carboxy-terminal processing proteases (CTPs). This novel group of serine proteases cleave at the very C-terminal of their substrate peptides. During this research mutant strains of *P.aeruginosa* with inactivated CTP genes *ctpA* and *prc* showed reduced secretion of major virulence factors such as Pseudolysin, Staphylolysin, Alkaline protease, Protease IV and the Aminopeptide PepB as analyzed by 2D gel electrophoresis. The results were confirmed by enzyme specific assays. These mutants were tested for virulence in four different infection models: Thale cress (*Arabidopsis thaliana*), Fruit fly (*Drosophila melanogaster*), *Caenorhabditis elegans* and the Greater wax moth (*Galleria Mellonella*). Results of the *A.thalinana* assay, the *D.melanogaster* feeding assay and the *C.elegans* slow killing assay showed reduced virulence of both the ΔctpA and Δprc mutant strain. In three other assays the virulence of both the ΔctpA and Δprc strains were tested in models with direct systemic infection by injection. The needle pricking assay in *D.melanogaster* showed difference in survival prolongation for the ΔctpA and Δprc strain. And within the *G.mellonella* injection assay both Δprc and the ΔctpA strain showed a significant difference in LD_{50} values. In this assay the Δprc was less virulent than the ΔctpA strain. Also was the lethally of the sterile supernatant fractions of the mutants tested by injection of
**Summary**

*G. mellonella.* The injection of the extracellular fractions of both ∆prc and the ∆ctpA cultures showed a longer survival time when compared to the wild type.

In order to further characterize the physiological function of CtpA and Prc in *P. aeruginosa* the CTPs were cloned, expressed and purified. The expression in *E. coli* seems to possible only under mild conditions such as a temperature of 24 °C and strict control of promoter leakage. The CTPs were shown to be active within a β-casein assay at pH 8.0 and 37°C. Alanine substitution mutation of the putative active residues serine and lysine revealed to be essential for activity for both CtpA as Prc. The active residues seem to form a catalytic dyad within the mechanism of peptide hydrolysis.

Penicillin-binding proteins 3 were suggested as physiological substrates of CTPs within bacteria. Genome mining revealed two possible PBP3 protein within *P. aeruginosa*. Both CtpA as Prc were able to cleave synthetic peptides mimicking the C-terminus of Penicilline Binding Proteins of *P. aeruginosa*. CtpA was able to cleave both PBP3 and 3A. Prc was able to cleave only PBP3A. It seems that not one specific protease is active against one distinct PBP3. Nevertheless the results present strong evidence that PBP3 and 3A are physiological substrates of CTPs. PBP3s play an important role during septation within the process of cell wall synthesis. The validity of the use of the peptides is being confirmed by the fact that a Prc cloned from *E. coli* was shown to be able to cleave the PBP3 synthetic peptide of *E. coli* at the proposed cleavage site.

CtpA and Prc were also able to process a synthetic peptide mimicking the peptide tag of the tmRNA tagging system. This system tags incorrectly synthesized protein in the ribosomes and directs them for degradation.

Bacterial CTPs show a high sequence similarity with CTP proteases from phototrophic organisms such as plants, algae and cyanobacteria. These proteases play an important role in the activation by cleavage of a short peptide from the C-terminal of the D1 precursor protein. The D1 protein is one of the major components of Photosystem II. Both CtpA and Prc were able to cleave synthetic peptides mimicking the C-terminal portion of the D1 precursor
proteins from Spinacia oleracea (Spinach) and Scenedesmus obliquus (green algae) at their native cleavage site. These results confirm their high mutually evolutionary relation.

The overall results presented in this thesis lead to the statement that CTPs are novel targets for the development of antibiotics. In order to facilitate the development of protease inhibitors a High Throughput screening assay was developed using a synthetic peptide with a fluorescent/quencher couple. The assay revealed two compounds Rhododime A and B as novel inhibitors of CtpA of *P. aeruginosa* and Prc of *E. coli*. These compounds are regarded as new lead compounds for the development of bacterial CTP inhibitors.
Zusammenfassung


Als physiologische Substrate für CTP’s innerhalb der Zelle werden Penicillin-bindende Proteine der Klasse 3 (PBP3) angenommen. PBP3 spielen eine wichtige Rolle bei der Bildung von Zellkompartmenten während des Prozess der Zellwandsynthese.

Peptid-tag des tmRNA tagging system nachbildet, zu prozessieren. Dieses System markiert fehlgefaltene Proteine in den Ribosomen und sorgt dafür, dass diese abgebaut werden.

Bakterielle CTP’s zeigen eine starke Sequenzähnlichkeit zu CTP’s aus phototrophen Organismen wie Pflanzen, Algen und Cyanobakterien. Dort spielen diese Proteasen eine entscheidende Rolle bei der Aktivierung des D1 Präkursor Proteins durch Abspaltung eines kurzen Peptids am C-Terminus dieser Proteine. Das D1 Protein ist eines der wichtigsten Proteine innerhalb des Photosystems II.

Sowohl CtpA als auch Prc waren in der Lage Peptide zu spalten, welche dem C-Terminus des D1-Präkursor Proteins aus Spinacia oleracea (Spinat) und Scenedesmus obliquus (Grünalge), also den natürlichen Substraten der CTP’s aus diesen Organismen, ähneln. Diese Resultate bestätigen die große evolutionäre Verwandtschaft von Vertretern der CTP-Enzymfamilie aus unterschiedlichen Organismen.

Die Ergebnisse dieser Arbeit deuten darauf hin, dass die P. aeruginosa CTP’s aufgrund der verringerten Sekretion der Virulenzfaktoren und der damit zusammenhängenden in Modellsystemen nachgewiesenen verringerten Virulenz von CTP-defizienten P. aeruginosa-Stämmen einen vielversprechenden neuen Angriffspunkt für neu zu entwickelnde Antibiotika darstellen. Um die Entwicklung von Protease-Inhibitoren zu erleichtern, wurde hier eine Hochdurchsatz-Selektionmethode entwickelt, mittels derer der Abbau von synthetischen Peptiden über Fluoreszens-(Quenching) - verfolgt werden kann. Mittels dieses Verfahrens konnten mit Rhododime A und B zwei Verbindungen als neue Inhibitoren von CtpA aus P. aeruginosa und Prc aus E.coli identifiziert werden. Diese könnten als Grundstruktur für die Entwicklung neuer Inhibitoren von bakteriellen CTPs herangezogen werden.
Samenvatting

Sinds de ontwikkeling en het intensieve gebruik van antibiotica vanaf het begin van de 20ste eeuw ontstond tevens het probleem van de resistentie ontwikkeling van deze middelen. Omdat de ontwikkeling en introductie van nieuwe antibiotica al jaren daalt is het probleem van multi-drug resistentie alarmerender dan ooit. De werking van de huidige antibiotica is erop gericht om de pathogene bacterie te vernietigen danwel zijn groei te remmen wat leidt tot een hoge selectie druk en daarmee de ontwikkeling van resistentie. Het doel van dit promotieonderzoek was het vinden van nieuwe aangrijpingspunten voor de ontwikkeling van antibiotica die erop gericht is de pathogeen alleen te ontwapenen waardoor het zijn virulentie verlies en op deze manier de selectie druk te verminderen en daarmee de ontwikkeling van resistentie.

*Pseudomonas aeruginosa* is in dit onderzoek gebruikt als het model organisme. *P.aeruginosa* is een aerobe Gram-negatieve bacterie en opportunistisch pathogeen en een belangrijke veroorzaker van zowel nosocomiale als niet-nosocomiale infecties. Vooral in patiënten met *Cystic Fibrosis* zorgt *P.aeruginosa* voor een hoge morbiditeit en mortaliteit.

De focus van dit onderzoek lag bij de groep van Carboxy-terminal processing proteases (CTPs). Deze nieuwe groep van serine proteasen snijden aan het uiterste Carboxylterminus van substraat peptiden. Tijdens het onderzoek lieten mutante stammen van *P.aeruginosa* waarbij de CTP genen zijn geïnactiveerd een verminderde uitscheiding van belangrijke virulentie factoren zoals Pseudolysine, Staphylolysine, Alkalische protease, Protease IV en het Aminopeptidase PepB na analyse van 2D gel electroforese (proteomics).

Ook bij het *G. mellonella* injectie experiment werd een significant verschil gezien in de LD$_{50}$ waarden. In dit experiment was $\Delta$prc minder virulent dan $\Delta$ctpA. Daarnaast is de letaliteit onderzocht van het steriele supernatant fracties van culturen van beide mutanten en geïnjecteerd in *G. mellonella*. De injectie van de extracellulaire fracties van zowel $\Delta$ctpA als $\Delta$prc lieten een langere overlevingstijd zien ten opzichte van het wildtype.

Om de fysiologische functies van CtpA en Prc in *P. aeruginosa* te onderzoeken zijn deze geklonen, tot expressie gebracht en gezuiverd. De overexpressie van deze CTPs bleek alleen mogelijk bij milde condities zoals een temperatuur van 24°C en onder strikte controle van promotor lekkage. De CTPs vertoonden activiteit in een experiment waarbij β-caseïne werd gesplitst bij een pH van 8.0 en 37°C. Alanine substitutie mutering van de vermoedelijke actieve residuen serine en lysine in het actieve centrum lieten zien dat deze essentieel zijn voor activiteit. De actieve residuen vormen vermoedelijk een katalytische diade binnen het mechanisme van de peptide hydrolyse.

Er is gesuggereerd dat Penicillin-binding proteins 3 eiwitten in bacteriën mogelijke fysiologische substraten zijn van CTPs. Na Genome mining werden 2 mogelijke PBP3 eiwitten (PBP3 en PBP3A) ontdekt in *P. aeruginosa*. Activiteit van CtpA en Prc werd aangetoond na splitsing van synthetische peptiden die de C-terminale einden nabootsten van PBP3 en PBP3A. Prc was instaat om alleen PBP3 te splitsen. CtpA splitste zowel PBP3 als PBP3A. Het lijkt erop dat niet één specifieke protease één PBP3 eiwit splitst. Desondanks laten de resultaten zien dat er sterk bewijs is dat PBP3 eiwitten fysiologische substraten zijn voor CTPs. PBP3 eiwitten spelen een belangrijke rol bij de vorming van het septum binnen het algehele proces van celwandsynthese.

CtpA en Prc waren ook instaat om een synthetisch peptide te splitsen dat het peptide label nabootste van het tmRNA labeling systeem. Dit systeem labelt incorrect gesynthetiseerde eiwitten in de ribosomen en leidt deze naar afbraak.

CTPs uit bacteriën hebben een grote gelijkenis met CTP proteases van fototrofe organismen zoals planten, algen en cyanobacteriën. Deze proteasen spelen een belangrijke rol in de activatie door splitsing van een klein peptide van het C-terminale einde van het D1 voorloper.
eiwit. Het D1 eiwit is een van de belangrijkste componenten van fotosysteem II. Zowel CtpA als Prc waren in staat om een synthetisch peptide te splitsen dat het C-terminale einde van het D1 voorloper eiwit nabootste van zowel Spinacia oleracea (Spinazie) als Scenedesmus obliquus (groen alg). De resultaten bevestigen de grote onderlinge evolutionaire gelijkenis.

De resultaten van een verminderde secretie van de virulentie factoren en verminderde virulentie binnen verschillende infectie modellen leiden tot de uitspraak dat CTPs nieuwe targets zijn voor de ontwikkeling van antibiotica. Om verdere ontwikkeling van potentiële remmers van deze proteasen te faciliteren is een High Troughput screenings assay ontwikkeld wat gebruikt maakt van een synthetisch peptide met fluoriserende/quencher koppel. Met behulp van deze assay zijn Rhododimer A en B geïdentificeerd als mogelijke remmers van CtpA uit P.aeruginosa als Prc uit E.coli. Deze twee stoffen worden beschouwd als nieuwe lead compounds in de ontwikkeling van bacteriële CTP remmers en dus nieuwe antibiotica.
THE BEGINNING

"It all starts with one simple stripe"
CHAPTER 1
Introduction

General Introduction
Scope of the thesis
Chapter summary

Since the development and intensive use of antibiotic drugs starting at the beginning of the 20th century also the problem of resistance towards these agents has emerged. As the development and introduction of novel antibiotics have declined over the years the problem of multi-drug resistance is becoming alarming by the day.

*Pseudomonas aeruginosa* is a good example of such a pathogen in which multi drug resistance has emerged. *P.aeruginosa* is an aerobic Gram-negative bacterium and opportunistic pathogen that is an important cause of both community-acquired and hospital-acquired infections. Especially in patients with Cystic Fibrosis *P.aeruginosa* causes high morbidity and mortality. Bacterial pathogens have two distinct abilities that makes them virulent. First their ability to secrete virulence factors which facilitates an infection and second their ability to form biofilms which enables them to protect themselves.

In this thesis the research was focused on the group of proteases as potential targets for antibiotics. Proteases are enzymes that enable the cleavage of peptides and proteins by hydrolysis of a peptide bond. Proteases can be divided into 6 different catalytic types: aspartic-proteases, metallo-proteases, serine-proteases, cysteine-proteases, threonine-proteases and glumatic-proteases depending on their active residues that are involved in the catalysis. The genome of *P.aeruginosa* comprises 5568 genes of which 155 genes are predicted proteases. This is about 2.8% of the genome.

The mechanism of today's antibiotics is to destroy or inhibit growth of the pathogenic bacterium which leads to high selection pressure and development of resistance. The aim of this thesis was to identify novel targets for the development of antibiotics that only disarm the pathogen in order to reduce its virulence and by doing so reducing the selection pressure and development of resistance.
General Introduction

The era of modern antibiotics started with the discovery of arsphenamine (arsenobenzol, Salvarsan or “606”) by Sahachiro Hata and Paul Ehrlich. The discovery was made in 1909 in Erlich’s laboratory after a structural and reconceived plan after which arsphenamine showed antisyphilitic activity (Sader et al., 2011). It was the most used antibiotic drug worldwide until 1945 after the discovery and following extensive use of penicillin (Zaffiri et al., 2012). With the arrival of antibiotics the survival of until then frequently deathly infectious diseases increased dramatically (Spellberg, 2014).

As the use of antibiotics gradually grew over the years and more and more antibiotics were developed also the amount of drug resistance emerged (Zaffiri et al., 2012). Figure 1.1 shows a graph of the amount of unique β-lactamase enzymes identified every year over the last half century (Davies and Davies, 2010). The graph shows the exponential increase of resistance over the years. The occurrence of antibiotic drug resistance or even multidrug resistance of truly pan-resistant pathogens such as Acinetobacter baumannii, Burkholderia cepacia, Campylobacter jejuni, Citrobacter freundii, Clostridium difficile, Enterobacter spp., Enterococcus faecium, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella spp., Serratia spp., Staphylococcus aureus, Staphylococcus epidermidis, Stenotrophomonas maltophilia, and Streptococcus pneumonia are major concerns in clinical microbiology (Davies and Davies, 2010; Payne, 2008).

This wouldn’t be a problem if mankind had an endless supply of effective antibiotics. Which is not the case. In the last centenary over 140 antibiotics has been discovered and developed for use in mankind (Spellberg, 2014). Although a decline of newly introduced and approved antibiotics has started since the 1980's. Figure 1.2 shows the development of new antibacterial drug approvals by the U.S. Food and Drug Administration (FDA). In period 2005-2009 only 3 new antibiotics were approved by the FDA. In the near future a small armory of new antibiotic drug may enter the drug market. Table 1 shows an inventory of new antibiotic drug approvals and compounds that in different phases of clinical trial.
Figure 1.1: Numbers of unique β-lactamase enzymes identified since the introduction of the first β-lactam antibiotics (Davies, 2010).

Figure 1.2: Numbers of antibiotic drugs approved by the FDA over the last 25 years.
Table 1.1: New antibiotics approved and/or in development.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Antibiotic class</th>
<th>Spectrum of microbiological activity</th>
<th>Main clinical indication</th>
<th>Development phase</th>
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<td>BAL30072</td>
<td>Monocyclic β-lactam</td>
<td>MDR P. aeruginosa Acinetobacter including metallo-β-lactamases and enterobacteriaceae</td>
<td>NK</td>
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<tr>
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<td>Gram-positive, including MRSA</td>
<td>cSSTIs</td>
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<td>Quinolone</td>
<td>Gram-positive and Gram-negative</td>
<td>ophthalmicinfection</td>
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<td>RTI, UTI</td>
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<td>Polymyxin</td>
<td>MDR Gram-negative</td>
<td>NK</td>
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<td>Cephalosporin</td>
<td>Gram-positive</td>
<td>cSSTIs, CAP</td>
<td>Approved by FDA and EMA</td>
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<td>Cephalosporin + beta lactamase-inhibitor</td>
<td>MDR P. aeruginosand enterobacteriaceae, excluding metallo-β-lactamases</td>
<td>cUTI, cSSTI, VAP</td>
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<td>cSSTI, hospitalized CAP</td>
<td>III</td>
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<td>Cephalosporin + beta lactamase-inhibitor</td>
<td>Gram-negative</td>
<td>cIAI, cUTIs, HAP, VAP</td>
<td>III</td>
</tr>
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<td>Cethromycin</td>
<td>Ketolide</td>
<td>Gram-positive and Gram-negative</td>
<td>CAP</td>
<td>III</td>
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<td>Dalbavancin</td>
<td>Glycopeptide</td>
<td>Gram-positive</td>
<td>cSSTI</td>
<td>III</td>
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<td>Delafloxacin</td>
<td>Quinolone</td>
<td>Broad-spectrum including fluoroquinolone-resistant MRSA</td>
<td>cSSTI</td>
<td>II</td>
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<td>Doripenem</td>
<td>Carbenem</td>
<td>Gram-negative</td>
<td>cUTIs, cIAIs, HAP, VAP</td>
<td>Approved by FDA and EMA</td>
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<td>Eravacycline</td>
<td>Tetracycline</td>
<td>Gram-negative but not Pseudomonas</td>
<td>cIAI</td>
<td>II</td>
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<td>Quinolone</td>
<td>Enhanced Gram-positive activity including fluoroquinolone-resistant MRSA</td>
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<td>CAP</td>
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</tr>
<tr>
<td>Nemonoxacin</td>
<td>Quinolone</td>
<td>Gram-positive and Gram-negative</td>
<td>CAP</td>
<td>III</td>
</tr>
<tr>
<td>Omadacycline</td>
<td>Tetracycline</td>
<td>Gram-positive and Gram-negative</td>
<td>cSSTIs, CAP</td>
<td>III</td>
</tr>
<tr>
<td>Oritavancin</td>
<td>Glycopeptide</td>
<td>Gram-positive, including MRSA, VRSA, VRE</td>
<td>cSSTIs</td>
<td>III</td>
</tr>
<tr>
<td>Panipenem</td>
<td>Carbenem</td>
<td>Gram-negative and positive</td>
<td>cUTIs, RTI, obstetrical and gynaecological infections</td>
<td>III</td>
</tr>
<tr>
<td>Plazomycin</td>
<td>Aminoglycoside</td>
<td>MDR enterobacteriaceae and S. aureus, including aminoglycoside-resistant and metallo-β-lactamase producers</td>
<td>cUTI, cIAI</td>
<td>II</td>
</tr>
<tr>
<td>Radezolid</td>
<td>Ozaolidinone</td>
<td>Gram-positive</td>
<td>CAP, SSTI</td>
<td>II</td>
</tr>
<tr>
<td>Razupenem</td>
<td>Carbenem</td>
<td>Gram-negative and Gram-positive</td>
<td>cSSTIs</td>
<td>II</td>
</tr>
<tr>
<td>Solithromycin</td>
<td>Carbenem</td>
<td>Gram-positive</td>
<td>CAP</td>
<td>III</td>
</tr>
<tr>
<td>Teipenem/pivoxil</td>
<td>Carbenem</td>
<td>Gram-positive and Gram-negative</td>
<td>otorolaryngological/RTI</td>
<td>II</td>
</tr>
<tr>
<td>Tedisolidphosphate</td>
<td>Ozaolidinone</td>
<td>Gram-positive, including MRSA</td>
<td>cSSTIs</td>
<td>III</td>
</tr>
<tr>
<td>Telavancin</td>
<td>Glycopeptide</td>
<td>Gram-positive</td>
<td>cSSTI, HAP, VAP</td>
<td>Approved by FDA and EMA</td>
</tr>
<tr>
<td>Tomipenem</td>
<td>Carbenem</td>
<td>Gram-positive, including MRSA and Gram-negatives including ESBL-producing Enterobacteriaceae</td>
<td>cSSTIs, HAP</td>
<td>II</td>
</tr>
</tbody>
</table>

NK not known, RTI respiratory tract infections, cSSTI complicated skin and soft tissue infections, cIAI complicated intra-abdominal infections, CAP community acquired pneumonia, HAP hospital acquired pneumonia, VAP ventilator associated pneumonia, UTI urinary tract infections (Bassetti et al., 2013).
Although there are several compounds being tested the list does not contain new compounds with a novel chemical scaffold nor antibiotic drug target. Only compound BC-3781 is belonging to the relative new group of Pleuromutilins which have a novel chemical scaffold. Retapamulin was the first antibiotic drug as member of the Pleuromutilins that was approved for use in humans by the FDA and the European Medicines Agency (EMA) in 2007 (Novak and Shlaes, 2010).

The development of new antibiotics is therefore necessary. Novel antibiotic strategies have emerged based on the idea not to tackle essential functions and kill a pathogen as conventional antibiotics do, but intend to cripple a pathogen's ability to establish and maintain an infection (Hoge et al., 2011). One example is the cell-cell signaling of *P. aeruginosa* which has successfully been addressed by novel anti-infective compounds (Lesic et al., 2007). However, any other cellular component which is required for full functionality of a virulence pathway and that can be inactivated without perturbing bacterial viability *per se* can be a promising target to further develop selective anti-infective drugs.
Pathogenesis of bacteria

Nature is occupied by thousands of bacteria species. Only a relative small amount of bacteria is pathogenic. Bacteria are called pathogenic if the species causes disease in the host-organism. Bacteria have a few characteristics that make them pathogenic (Wilson et al., 2002). The pathogenic mechanisms can divided in two qualities; 1) the ability to invade and evade the host defense system and 2) the ability to adhere and establish an infection. Figure 1.3 gives an overview of the bacterial mechanisms for pathogenicity (Wilson et al., 2002).

An import feature of this pathogenesis is the ability to secrete virulence factors and/or toxins. These compounds may be of protein origin or non-protein (small molecular weight compounds). These secreted compounds may interfere with several aspects during infection such as help to evade host defense mechanism (e.g. exopolysacharides, or proteases) or to disrupt the host physiology or damages the host such as toxins (e.g. LPS, or exotoxins) (Wilson et al., 2002).

Another feature of some pathogenic pathogens is their ability to form biofilms. Biofilms formation enables single-cell organisms to assume a temporary multicellular lifestyle, in which “group behavior” facilitates survival in adverse environments (Kostakioti et al., 2013). In a biofilm adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS). The transformation of a platonic state to biofilm enables the bacteria to deal with more harsh conditions. The formation of biofilms by pathogenic bacteria makes it more difficult for antibiotics to reach their targets and is associated with more severe and chronic infections (Kostakioti et al., 2013).
Figure 1.3: An overview of bacterial mechanisms for pathogenicity. (A) Upon encountering a human host, a bacterial pathogen may illicit several host responses and use a variety of mechanisms to evade the host defences. The bacterial components that interact with the host include: (1) capsules that act to “frustrate” phagocytosis and protect the pathogen from macrophage and neutrophil engulfment, (2) lipopolysaccharide (LPS) and cell wall components which can cause septic shock, (3) toxins that can serve to damage host cells and aid invasion, and (4) adhesins which facilitate binding of the pathogen to host surfaces. The degree to which these various mechanisms play a part in the pathogenesis of an infection depends on the bacterial species or strain, the site of pathogen entry, the immune status of the host and other similar factors. (B) Once adhered to a host surface, a bacterial pathogen may further invade host tissues. Pathogens may “burrow” further into a tissue by expressing and secreting proteases and glycanases that digest host extracellular matrix proteins and polysaccharides. In addition, a pathogen may also invade the host tissue cells and gain access to the intracellular environment. This can be facilitated by the natural phagocytosis mechanisms of macrophages and neutrophils or by induced uptake where the pathogen signals the host cell to engulf adhered bacteria. A common strategy for pathogens to induce uptake is the use of a type III secretion system which injects bacterial signaling proteins into the host cell. Within the host cell, the pathogen may reside within a phagolysome (a phagosome which has fused with a lysosome), a phagosome which has not fused with a lysosome, or within the host cell cytosol. (Wilson et al., 2002).

\textit{Pseudomonas aeruginosa} (cited from Hoge et al., 2011)
*Pseudomonas aeruginosa* is an aerobic Gram-negative bacterium that is an important cause of both community-acquired and hospital-acquired infections (Driscoll et al., 2007). Community-acquired infections include among others ulcerative keratitis (usually associated with contact lens use), otitis externa, skin and soft tissue infections (including diabetic foot infections).

Hospitalized patients may be colonized with *P. aeruginosa* on admission or during hospital stay. Nosocomial infections include pneumonia, urinary tract infections, bloodstream infections, surgical site infections and infection of the skin after burn injuries. *P. aeruginosa* infections also occur in immunocompromised patients e.g. AIDS especially in patients who have a compromised phagocytic system (Lang et al., 2004).

*P. aeruginosa* is the leading cause of respiratory tract infections with patients which are intubated during a hospital stay and has a high mortality of 40% to 50% (Murray et al., 1999). Chronic sinopulmonary colonization and recurrent infections of *P. aeruginosa* are seen in patients with Cystic Fibrosis. In a survey in 2004 57.3% of all reported respiratory cultures from CF patients contained *P. aeruginosa* and in another survey 97.3% of CF children had a *P. aeruginosa* infection by the age of three (Driscoll et al., 2007). In Europe *P. aeruginosa* is the most prevailing bacterium that caused chronic lung infection in CF patients (European Cystic Fibrosis Society, 2007). CF lung infections are characterized by a vigorous inflammatory response with increased cytokine production including interleukin-8 (IL-8) and interleukin-6 (IL-6) and a neutrophil-dominant inflammatory response in the airways (Voynow et al., 2008). *P. aeruginosa* proteases are detected in the lungs of CF patients such as elastase B, alkaline protease, protease IV and PasP (Suter et al., 1984; Uritchard et al., 2008).

Wound infections due to *P. aeruginosa* are especially difficult in burn patients (Murray et al., 1999). A high percentage of the wound infections will lead to sepsis with significant mortality rates. Protease-deficient strains are generally less virulent than protease producers in burned mouse models (Holder and Haidaris, 1979). *P. aeruginosa* infections of the eye usually follows minor trauma to the cornea (Murray et al., 1999). These infections are frequently associated with contact lens use. Proteases play an important role during infection with *P. aeruginosa*.
and are a characteristic for invasiveness as determined in clinical strains (Janda and Bottone, 1981).

**Proteases** (cited from Hoge et al., 2011)

Proteases or peptidases are enzymes that can hydrolyze peptide bonds within peptides and proteins (Barrett et al.). For a long time the function of proteases were solely dedicated to protein turnover or digestion of proteins as a food source. More and more it becomes clear that the hydrolysis of a peptide bond of proteins can have a wide range of biological functions which can be very subtle and specific.

Proteases can be divided into 6 different catalytic types: aspartic-proteases, metallo-proteases, serine-proteases, cysteine-proteases, threonine-proteases and glutamic-proteases depending on their active residues that are involved in the catalysis. The aspartic, metallo and serine-proteases are definitely the most abundant protease groups (Rawlings et al., 2010).

Genomes of different *P. aeruginosa* strains are available and several of them can be found in the MEROPS peptidase database. The well known PAO1 laboratory strain of *P. aeruginosa* contains about 5568 predicted genes (Stover et al., 2000). Of these genes 155 are predicted proteases and deposited in the MEROPS database (Rawlings et al., 2010). This is about 2.8% of the genome. The proteases are classified in the different catalytic types: 84 serine-proteases (54% of total proteases), 45 (29%) metallo-proteases, 11 (7%) cysteine-proteases, 5 (3%) threonine-proteases, 3 (2%) aspartic-proteases and 7 (5%) unassigned proteases.
Scope of the thesis

The identification of novel targets for the development of new antibiotic drugs is necessary to win the combat from infectious diseases and the ever increasing problem of multi-drug resistance. The results of this thesis is part of research executed within the ANTIBIOTARGET consortium. The ANTIBIOTARGET consortium is a collaboration network involving several European scientific institutes: Universität Ulm (Ulm, Germany); Heinrich-Heine Universität (Düsseldorf, Germany); University of Nottingham (Nottingham, United Kingdom), Imperial College (London, United Kingdom), Rijksuniversiteit Groningen (Groningen, the Netherlands) and Institut de Microbiologie de la Méditerranée-CNRS (Marseille, France).

The aim of this thesis was to identify novel bacterial proteases as antibiotargets for infections of *Pseudomonas aeruginosa* and to develop inhibitors. Therefore bacterial proteases had to be identified as possible targets by proteomic techniques. Identification needed to be proven by specific assays. Targets needed to be localized within the organism. Potential targets had to be tested in animal infection models. Targets had to be cloned, expressed and characterized. Target assays had to be developed to show activity and the physiological function had to be elucidated. Small molecular compounds had to be tested as possible inhibitors of the targets.
References


"At the earliest drawings of the fractal curve, few clues to the underlying mathematical structure will be seen."

IAN MALCOLM

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff*
CHAPTER 2

Materials and Methods
Materials and Methods

Bacterial strains

Bacterial strains used within this thesis are listed in table 2.1.

<table>
<thead>
<tr>
<th>Table 2.1: Bacterial strains used in this thesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
</tr>
<tr>
<td>PAO1</td>
</tr>
<tr>
<td>PAM6</td>
</tr>
<tr>
<td>PAM7</td>
</tr>
<tr>
<td>K12-MG1655</td>
</tr>
<tr>
<td>S71.1</td>
</tr>
<tr>
<td>DH5α</td>
</tr>
<tr>
<td>BL21(DE3)</td>
</tr>
<tr>
<td>OP50</td>
</tr>
<tr>
<td>JM109(DE3)</td>
</tr>
</tbody>
</table>
Chapter 2

Vectors

Vectors used within this thesis are listed in table 2.2.

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBBR1-MCS1</td>
<td>rep mob lacZa Plac PT7 CmR</td>
<td>(Kovach et al., 1995)</td>
</tr>
<tr>
<td>pCtpA-lac</td>
<td>Amplicon of a region covering ORF PA5134, without the native promoter but maintaining the ribosome-binding site with Fw-ctpA-lac and Rv-ctpA-lac. Cloned into pBBR1-MCS1 over EcoRV.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pET-19b</td>
<td>Cloning plasmid with N-terminal His-tag followed by an enterokinase cleavage site and three cloning sites, T7 expression system, pBR322 origin, bla antibiotic resistance gene, 5717 bp.</td>
<td>Novagen, Darmstadt Germany</td>
</tr>
<tr>
<td>pET-19b-CtpA</td>
<td>1,265 bp amplicon with Fw-ctpA19b and Rv-ctpA19b carrying the entire PA5134 ORF (bases 5782278 to 5783588) of PAO1 cloned into pET-19b using Ndel/Xhol.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pET-19b-Prc</td>
<td>2,051 bp amplicon with Fw-prc19b and Rv-prc19b carrying the entire PA3257 ORF (bases 3642964 to 3645060) of PAO1 cloned into pET-19b using Ndel/Xhol.</td>
<td>This thesis</td>
</tr>
<tr>
<td>pET-19b-PrcEc</td>
<td>2,049 bp amplicon with Fw-prcEc19b and Rv-prcEc19b carrying the entire b1830 ORF (bases 1910792 to 1912840) of K12-MG1655 cloned into pET-19b with Ndel/Xhol.</td>
<td>This thesis</td>
</tr>
<tr>
<td>materials and Methods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>pET19-b-CtpASA</strong></td>
<td>1,265 bp amplicon with <em>w-ctpA</em>19b and <em>Rv-ctpA</em>19b and fragments 1 and 2 as template and cloned into pET19b using NdeI/Xhol. Fragments 1 and 2 were amplified with Fw-ctpASA and ctpAS301A and ctpAS301A and Rv-ctpASA, respectively using pET19b-CtpA as template.</td>
<td></td>
</tr>
<tr>
<td><strong>pET19b-CtpAKA</strong></td>
<td>1,265 bp amplicon with Fw-ctpA19b and Rv-ctpA19b of fragments 3 and 4 as template and cloned into pET19b using NdeI/Xhol. Fragments 3 and 4 were amplified with Fw-ctpAKA and ctpAK327A and ctpAK327A and Rv-ctpAKA , respectively using pET19b-CtpA as template.</td>
<td></td>
</tr>
<tr>
<td><strong>pET-19b-PrcSA</strong></td>
<td>2,049 bp amplicon with Fw2 and Rv2 of fragments 5 and 6 as template and cloned into pET19b using NdeI/Xhol. Fragments 5 and 6 were amplified with Fw-prcSA and S479A and S479A and Rv-prcSA , respectively using pET-19b-Prc as template.</td>
<td></td>
</tr>
<tr>
<td><strong>pET-19b-PrcKA</strong></td>
<td>2,049 bp amplicon with Fw2 and Rv2 of fragments 7 and 8 as template and cloned into pET19b using NdeI/Xhol. Fragments 7 and 8 were amplified with Fw-prcKA and prcK504A, and prcK504A and Rv-prcKA, respectively using pET-19b-Prc as template.</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

Oligonucleotides

Oligonucleotides/primers were synthesized and purchased from Eurofins MWG Operon (Ebersberg, Germany) as lyophilisate of HPSF® purity. The oligonucleotides were dissolved in demineralized water. Melting temperatures ($T_m$) were calculated by the manufacturer. Table 2.3 gives an overview and sequences of all used oligonucleotides within this thesis.

<table>
<thead>
<tr>
<th>Label</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fw-ctpA-lac</td>
<td>GCC GAA CTC GTG ATT AGG AGC</td>
<td>Constitute expression CtpA in <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Rv-ctpA-lac</td>
<td>GTT GAA CAG CAG GCA CAG G</td>
<td>Constitute expression CtpA in <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Fw-ctpA19b</td>
<td>TTT TTC ATA TGG CCG ACG CCC CGG</td>
<td>Overexpression CtpA from <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Rv-ctpA19b</td>
<td>TTT CTC GAG TTA TCA GTT GCC GCG GGT GAC</td>
<td>Overexpression CtpA from <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Fw-prc19b</td>
<td>TTT TTC ATA TGT CGA CCG CCG TCT CCG TC</td>
<td>Overexpression Prc from <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Rv-prc19b</td>
<td>TTT CTC GAG TTA TCA GTG CTT GGC CAC CG</td>
<td>Overexpression Prc from <em>P. aeruginosa</em></td>
</tr>
<tr>
<td>Fw-prcEc19b</td>
<td>TTT TTC ATA TGG TAG AAG ATA TCA CGC G</td>
<td>Overexpression Prc from <em>E. coli</em></td>
</tr>
<tr>
<td>Rv-prcEc19b</td>
<td>TTT CTC GAG TTA TTA CTT GAC GGG AGC GG</td>
<td>Overexpression Prc from <em>E. coli</em></td>
</tr>
<tr>
<td>ctpAS301A</td>
<td>GT GGC AGC GCC TCG GCG GCG GAA ATC</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.3: Oligonucleotides used in this thesis*
<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Amino acid exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fw-ctpASA</td>
<td>GT GGC AGC GCC GCG GCG GCG GAA ATC</td>
<td>(S302A) (inactivation) CtpA</td>
</tr>
<tr>
<td>Rv-ctpASA</td>
<td>GAT TTC CGC CGC CGC GGC TGC GCC AC</td>
<td></td>
</tr>
<tr>
<td>ctpAK327A</td>
<td>GAC AGC TTC GGC AAG GGC TCG GTG CAG</td>
<td></td>
</tr>
<tr>
<td>Fw-ctpAKA</td>
<td>GAC AGC TTC GGC GCG GGC TCG GTG CAG</td>
<td>(K327A) (inactivation) CtpA</td>
</tr>
<tr>
<td>Rv-ctpAKA</td>
<td>CTG CAC CGA GCC CGC GCC GAA GCT GTC</td>
<td></td>
</tr>
<tr>
<td>prcS479A</td>
<td>GT CTC TCG GCC TCC GCC TCG GAG ATC</td>
<td>(S479A) (inactivation) Prc</td>
</tr>
<tr>
<td>Fw-prcSA</td>
<td>GT CTC TCG GCC GCC GCC TCG GAG ATC</td>
<td></td>
</tr>
<tr>
<td>Rv-prcSA</td>
<td>GAT CTC CGA GCC GCC CGA GAG AC</td>
<td></td>
</tr>
<tr>
<td>prcK504A</td>
<td>CAG ACC TTC GGC AAG GCC ACC GTG CAG</td>
<td></td>
</tr>
<tr>
<td>Fw-prcKA</td>
<td>CAG ACC TTC GGC GCG GCC ACC GTG CAG</td>
<td>(K504A) (inactivation) Prc</td>
</tr>
<tr>
<td>Rv-prcKA</td>
<td>CTG CAC GGT GCC CGC GCC GAA GGT CTG</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2

Chemicals

Chemicals of adequate quality were purchased from the following vendors.

Carl Roth (Karlsruhe, Germany)

Agar-agar, ammonium bicarbonate, ammonium sulfate, ampicillin, ethidiumbromide, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, calcium chloride, glycerine, isopropyl β-D-1-thiogalactopyranoside, low-fat skim milk, glucose, lactose, manganese (II) chloride, magnesium sulphate, magnesium chloride, monopotassium phosphate, Peptone, potassium acetate, potassium chloride, rubidium chloride, sodium chloride, sodium hydrogen phosphate, tromethamine (Tris), Tryptone, Yeast extract

Calbiochem (Darmstadt, Germany)

Z-Arg-Arg-pNA.2HCl

Fermentas (St. Leon-Rot, Germany)

T4 DNA ligase
Ndel (10 U/µL)
Xhol (10 U/µL)

Fisher Scientific (Schwerte, Germany)

Acetonitrile

GE Healthcare Bio-Sciences AB (Upppsala, Sweden)

IPG Buffer pH-3-11

New England Biolabs (Ipswich, USA)

Phusion® polymerase, GC buffer

Promega (Madison, USA)

Trypsin Gold®

Sigma-Aldrich (St. Louis, USA)

Betaïne, carbenicillin, chloramphenicol, Elastin Congo Red, formic acid, gentamycin, L-Leucine-4-nitroanilide, trichloroacetic acid, triclosan, N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate (Chromozym PL)
Cultivation and storage of bacteria

Growth medium
The ingredients were mixed and dissolved and stored in appropriate flasks. The prepared growth medium was directly sterilized at 121 °C and 200 kPa for 15 minutes. When desired appropriate additives (see table 2.4) such as antibiotics or X-gal were added afterwards to the medium (< 50°C) by sterile handling and filtered through a sterile filter of 0.2 μm (PVDF, VWR International GmbH, Darmstadt, Germany).

Luria-Bertani (LB)-liquid broth (Miller, 1972)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>ad 1000 ml</td>
</tr>
</tbody>
</table>

Total volume 1000 ml

The pH was adjusted to 7.0 with 5 N NaOH solution. The mixture was sterilized and stored at room temperature.

Luria-Bertani (LB)-solid broth (Miller, 1972)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>15.0 g  (1.5%)</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>ad 1000.0 ml</td>
</tr>
</tbody>
</table>

Total volume 1000.0 ml
The pH was adjusted to 7.0 with 5 N NaOH solution. The mixture was sterilized and when needed additives were added. Subsequently Petri-dishes were pored under sterile conditions. The broth was solidified and stored at room temperature.

**Luria–Bertani (LB) - Auto Induction broth (Studier, 2005)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>3.3 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>6.8 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>7.1 g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>ad 1000.0 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.0 with 5 N NaOH solution. The mixture was sterilized and when needed additives were added.
Materials and Methods

Super Optimal Broth with Catabolite repression (SOC) (Hanahan, 1983)

Trypton  
Yeast extract  
NaCl  
KCl 0.25 M solution in water  
MgCl\textsubscript{2} 2 M solution in water  
Glucose  
Demineralised water

<table>
<thead>
<tr>
<th>Substance</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 µg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>-</td>
<td>600 µg/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50 µg/ml</td>
<td>300 µg/ml</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10 µg/ml</td>
<td>30 µg/ml</td>
</tr>
<tr>
<td>IPTG</td>
<td>0.1 mM</td>
<td>-</td>
</tr>
<tr>
<td>Triclosan</td>
<td>-</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>X-gal</td>
<td>160 µg/ml</td>
<td>-</td>
</tr>
</tbody>
</table>

The trypton, yeast extract, NaCl and glucose was dissolved together in 200 ml water. Subsequently KCl and MgCl\textsubscript{2} solutions were added and mixed. Demineralised water was added to 250 ml and the pH adjusted with 5 N NaOH to 7.0. The broth was sterilized for 15 min. at 121 °C and stored at room temperature.

Cultivation of bacteria general

Precultures of E.coli and P.aeruginosa strains were grown overnight (16 hours) after inoculation with a single colony by a sterile wooden toothpick in 5 ml LB liquid medium in glass tubes and grown in a rotator at 40 rpm at 37 °C.

For higher volume cultures were grown in sterile Erlenmeyer flasks (volumes 10 fold of the grown medium volume) and inoculated by a single colony or 5 µl of a -80 °C stock culture. The cultures were grown in an incubator agitating on a shaker at 150 r.p.m. in an aerobic
Atmosphere. Strains were grown at 37 °C or other temperatures will stated accordingly. Main cultures were inoculated to a OD$_{580}$ of 0.05.

**Cultivation of bacteria for 2D gel electrophoresis and enzyme assays**

*P. aeruginosa* PAO1 wild type and ΔctpA and Δprc mutant strains were inoculated from overnight precultures to an OD$_{580}$ of 0.05 in 20 ml LB broth and grown at 37 °C, on an agitating shaker at 150 r.p.m. to an OD$_{580}$ of 0.5 in about 3 hours. Main cultures were inoculated in 25 ml LB to an OD$_{580}$ of 0.005 and grown for 16 hours under the same conditions followed by supernatant preparation.

**Cultivation of bacteria for infection assays of A.thaliana, D.melanogaster and C.elegans**

The strains *P. aeruginosa* PAO1 wild type, ΔctpA and Δprc mutant strains were grown overnight in LB broth at 37 °C, on an agitating shaker at 150 r.p.m. The next morning strains were inoculated at OD$_{580}$ 0.05 in 20 ml LB broth at 37 °C and grown to an O.D.$_{580}$ of 3.0.

**Storage of bacteria**

Bacterial strains were stored for longer periods at -80 °C. Therefore 150 µl DMSO was added to 1.5 ml of bacterial suspension in LB broth.

**Transformation of bacteria with vectors**

**Chemical competent cells preparation**

Chemical competent cells were prepared by the methods of Mülhart, 2003. A preculture of the desired *E.coli* strains were grown overnight at 37 °C. Subsequently 5x 50 ml LB broth was inoculated with 50 µl of the overnight *E.coli* culture and was grown to an OD$_{595\text{nm}}$ of 0.4-0.7 (optimal. 0.6) in about 4 hr at 37 °C. After the cultures reached the optimal OD, they were transferred to sterile 50 ml tubes. The bacteria were kept on ice between handling. The tubes were centrifuged for 15 min. at 3000g and 4 °C. The supernatant was discarded after centrifuging and 30 ml of sterile TFB I solution was added to each pellet. The pellets were resuspended by vortexing and incubated on ice for 15 min. Subsequently the tubes were centrifuged for 15 min. at 3000g and 4 °C to spin down the bacteria. Afterwards the
Materials and Methods

supernatant was discarded. 4 ml of TFB II solution was added to each tube and the pellet resuspended. All suspensions were combined and mixed. 200 µl aliquots from the suspension were made in 1,5 ml sterile microcentrifuge tubes and frozen immediately with liquid nitrogen and stored at -80 °C.

**TBF I solution (Mülhart, 2003.)**

Calcium chloride 0.666 g (f.conc. 10 mM)
Potassium acetate 1.767 g (f.conc. 30 mM, pH 5.8)
Rubidium chloride 7.255 g (f.conc. 100 mM)
Manganese (II) chloride 3.775 g (f.conc. 50 mM)
Glycerine 113.517 g (f.conc. 15% (v/v))
Demineralised water ad 600 ml
Total volume 600 ml

The potassium acetate was dissolved in 450 ml water. Rubidium chloride, the manganese chloride and calcium chloride were added subsequently. At last the glycerine was added. The volume was adjusted to 600 ml with water and the pH adjusted to 5.8. The solution was sterilized for 15 min. at 121 °C.

**TBF II solution (Mülhart, 2003.)**

Calcium chloride 0.832 g (f.conc. 75 mM)
MOPS 0.209 g (f.conc. 10 mM, pH 7.0)
Rubidium chloride 0.121 g (f.conc. 10 mM)
Glycerine 18.912 g (f.conc. 15% (v/v))
Demineralised water ad 100 ml
Total volume 100 ml
MOPS was dissolved in 75 ml water. Calcium chloride and rubidium chloride was added subsequently. Glycerine was added at last. The volume was adjusted to 600 ml with addition of water and the pH to 7.0. The solution was sterilized for 15 min. at 121 °C.

Transformation with chemical competent cells

About 1 ng of vector DNA was added to 200 μl of chemical competent cells. The mixture was incubated for 30 minutes on ice followed by a heat shock of 2 minutes at 42 °C. Thereafter 900 μl of SOC medium was added to the cells and grown for 60 minutes at 37 °C. The cells were centrifuged for 1 minute at 10,000g and 900 μl supernatant was discarded. The pellet was resuspended in the remaining supernatant, plated out on Petri-dishes with the appropriate medium and grown at 37 °C or room temperature until appearance of colonies.

Biparental filter matings (Windgassen et al., 2000)

Transformation of vectors to *P. aeruginosa* were executed by biparental filter matings. Therefore *E. coli* S17.1 donor strains with the desired vector were grown overnight in LB broth and appropriate selection pressure as well as a *P. aeruginosa* acceptor strain. Fresh cultures of the donor *E. coli* S17.1 strain were grown to within the logarithmic grow curve (OD$_{580}$ between 0.5 and 0.8) in 10 ml of LB broth. 1 ml over the overnight *P. aeruginosa* culture was incubated at 46 °C for 10 minutes to inactivate the restriction modification system. Followed by centrifugation of both 1 ml of the *P. aeruginosa* culture and 2 ml of the *E. coli* culture by centrifugation for 1 minute at 1,500g. The pellets were washed with 1 ml of a sterile 150 mM NaCl solution. The pellets were resuspended in 100 μl of the same solution after which both suspension of donor and acceptor were combined and mixed. The resulting suspension was plated out on a sterile conjugation filter on a LB-agar with triclosan and appropriate selection suppression. The plate was incubated at 37 °C overnight. After the incubation the filter is transferred to 2ml of liquid LB broth and resuspended. 100 μl of the suspension is again plated out on the same solid LB-agar as before and incubated.
Isolation of DNA

Genomic DNA from *P. aeruginosa* and *E. coli* was isolated with use of the DNeasy® Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according the instruction of the manufacturer. Plasmid and vector DNA was isolated depending of the required amount with the QIAprep® Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) or the NucleoBond® Xtra Midi Kits (Macherey-Nagel GmbH & Co. KG, Düren) according the instruction of the manufacturer.

DNA concentration was determined by measuring the absorbance at 260 nm with a BioPhotometer® (Eppendorf AG, Hamburg, Germany) and insertion of a TrayCell® curvette (Hellma Analytics GmbH & Co. KG, Müllheim, Germany). The DNA concentration was calculated using the extinction coefficient ε of 0.020 (μg/ml)⁻¹ cm⁻¹ for double-stranded DNA.

In vitro recombination of DNA

Hydrolysis of DNA was executed with different type-II restriction enzymes. For the ligation of DNA fragments T4 DNA Ligase was used. Both the use of the restriction enzymes and the ligase was performed according the instruction of the manufacturer (Fermentas GmbH, St. Leon-Rot, Germany). For purification of the DNA from enzymes and buffers the samples were purified using the QIAquick® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the instruction of the manufacturer.

Vectors were transformed to *E.coli* of *P. aeruginosa* as described above. When applicable transformed clones were screened using Blue-White Color selection. The transformants were plated on selective agar with both X-gal and IPTG. Plates were incubated overnight at 37 °C or at room temperature until colonies appeared. Colonies with plasmid that have an insert will remain white. Plasmid that don’t have a insert will turn blue. For a further blue color enhancement the plates were placed at 4 °C for 2 hr followed by overnight growth.
Polymerase Chain Reactions (PCR)

The amplification of DNA fragments was standard executed according to the following protocol:

<table>
<thead>
<tr>
<th>Component</th>
<th>type</th>
<th>amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC buffer 5x</td>
<td>reaction buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>Template</td>
<td>DNA</td>
<td>x μl</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>Deoxynucleotides</td>
<td>1 μl</td>
</tr>
<tr>
<td></td>
<td>(200 mM)</td>
<td></td>
</tr>
<tr>
<td>Forward primer</td>
<td>25 pmol (500 nM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>25 pmol (500 nM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Betaïn 5 M solution</td>
<td>Modifier</td>
<td>10.0 μl</td>
</tr>
<tr>
<td>Water</td>
<td>PCR grade</td>
<td>ad 50 μl</td>
</tr>
<tr>
<td>Phusion®</td>
<td>DNA polymerase</td>
<td>0.5 μl</td>
</tr>
<tr>
<td></td>
<td>1 unit</td>
<td></td>
</tr>
<tr>
<td>Total:</td>
<td></td>
<td>50 μl</td>
</tr>
</tbody>
</table>

The Phusion® High-Fidelity DNA Polymerase is a combination of a pfu polymerase with a processivity-enhancing domain.

The PCR reaction was performed using the Mastercycler® gradient PCR cycler (Eppendorf AG, Hamburg, Germany) using the following standard protocol:

**Amplification program**
- Lid 105 °C
  1. 98 °C for 180 s
  2. 98 °C for 30 s
  3. Tₐ for 30 s
  4. 72 °C for 50 s
  5. Repeat steps 2-4 30 cycles
  6. 72 °C for 10 min.
  7. 4 °C hold

After the run cups were cooled at 4 °C
The annealing temperature was calculated from the following formula:

\[ T_{a^{opt}} = 0.3 \times T_{m^{primer}} + 0.7 \times T_{m^{product}} - 14.9 \]

\( T_{a^{opt}} \) = optimal annealing temperature
\( T_{m^{primer}} \) = lowest melting temperature of the two primers
\( T_{m^{product}} \) = melting temperature of the product, which can be calculated with the formula:

\[ T_{m^{product}} = 81.5 + 16.6 \times (\log_{10}[J^*]) + 0.41 \times (GC\%) - (675/n) \]

\( GC\% \) = GC content
\( N \) = the number of base pairs of the product.
\( [J^*] \) = the concentration of monovalent cat-ions in the solution in mol/l.

After the PCR reaction amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the instruction of the manufacturer.

**Sequencing of DNA**

In order to verify the right sequence of the recombinant DNA fragments and vectors, these were sequenced by Eurofins MWG Operon (Ebersberg, Germany).

**Gel electrophoresis of DNA**

DNA vectors, PCR amplicons or restriction fragments were analysed with agarose gel electrophoresis. DNA samples were mixed 5:1 with DNA agarose loading solution and loaded into the agarose gel. The gel contained 1% (w/v) agarose in 0.5 fold TBE buffer. The 0.5 fold TBE buffer was also taken as running buffer for the electrophoresis. For visualisation of DNA after the run the agarose gel also contained 0.5 μg ethidium bromide per ml gel. The DNA was separated by using constant 120 V. Depending on the expected size of the DNA fragment
the 1 kb or 50 bp GeneRuler® DNA Ladder was used as marker (Fermentas GmbH, St. Leon-Rot, Germany). After the run the DNA was visualized by enlighten the gel with UV light (\( \lambda = 254 - 366 \text{ nm} \)) on a Eagle Eye II Imaging System using the IntasApp software (Stratagene GmbH, Heidelberg, Germany). DNA fragments of desire were cut from the gel and purified by using the QIAquick® Gel Extraction Kits (Qiagen GmbH, Hilden, Germany) according to the instruction of the manufacturer.

**DNA agarose loading solution 6x stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (f. conc.)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer 10 mM, pH7.6</td>
<td></td>
<td>4.0 ml</td>
</tr>
<tr>
<td>EDTA disodium dehydrate</td>
<td>(f.conc. 50 mM)</td>
<td>186 mg</td>
</tr>
<tr>
<td>SDS</td>
<td>(f.conc. 0.5% (w/v))</td>
<td>500 mg</td>
</tr>
<tr>
<td>Glycerine</td>
<td>(f.conc. 60% (v/v))</td>
<td>7.57 g</td>
</tr>
<tr>
<td>Bromophenol blue 1%</td>
<td>(f.conc. 0.03 %)</td>
<td>300 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>10 ml</td>
</tr>
</tbody>
</table>

**0.5 fold TBE buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (f. conc.)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>(f.conc. 35.6 mM)</td>
<td>4.3 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>(f.conc. 35.6 mM)</td>
<td>2.2 g</td>
</tr>
<tr>
<td>EDTA disodium dehydrate</td>
<td>(f.conc. 1 mM)</td>
<td>0.37 g</td>
</tr>
<tr>
<td>Demineralised water</td>
<td></td>
<td>ad 1000 ml</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td></td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Overexpression of protein

In vivo expression

Constructs were brought to overexpression in either E.coli strains JM109(DE3) or BL21(DE3) harboring a pET19b based vector. The pET19b vector has a T7 expression system and pBR322 origin. The vector contains a bla selection gene and the cloning site facilitate a N-terminal His-tag followed by an enterokinase cleavage site. Standard overexpression of constructs were performed as follows. Preculture of the overexpression strains were grown overnight at 37 °C in LB medium with ampicillin and additional 0.4% glucose to suppress promoter leakage. Expression cultures were grown under the same conditions after inoculation of OD$_{580nm}$ 0.05 to an OD$_{580nm}$ of 0.6 after which induction followed by addition of 1 mM IPTG. Cells were harvested after 3 hours. Protein production was analysed by SDS-PAGE.

In vivo overexpression was also executed with auto-induction medium. Here were precultures grown as described above and expression cultures were inoculated with OD$_{580nm}$ 0.05 in auto-induction medium. Expression cultures were grown for 16 hours at 37 °C.

In vitro expression

For in vitro expression of protein the EasyXpress® Protein Synthesis Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufactures instructions. In principle the kit contains a lysates that contains all translational machinery components (ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) as well as T7 and E. coli RNA polymerases. In this way the coupled transcription-translation system enables the expression of full-length proteins from T7 or E. coli promoters in a single-step reaction using plasmid or linear DNA templates.

Purification of proteases

After overexpression cells were harvested by centrifugation at 3000 × g for 10 minutes and suspended in resuspension buffer. The cell pellets were frozen at -20°C for at least 1 h and lysed using a French Press Cell Disrupter (Thermo Scientific, Waltham, USA). The lysate was
centrifuged at 20,000 × g for 20 minutes at 4°C. Between handling the purification fractions were kept on ice. The supernatant was loaded onto a 5 ml Nickel-nitrilotriacetic acid agarose column (Qiagen, Hilden, Germany). The purification was done according to the manufacturer's recommendations. In short, the column was equilibrated with resuspension buffer and washed with wash buffer and followed by elution with elution buffer. Centrifugal concentrators with polyethersulfone membranes and a molecular weight cut-off of 30 kDa (Sartorius AG, Goettingen, Germany) were used to change the buffer of the eluates to 50 mM Tris-HCl, pH 8.0. To these fractions which contained the purified protein, glycerol was added to a final concentration of 30% (v/v) before they were stored at -20°C. Protein concentration was determined with a BCA assay kit (Carl Roth, Karlsruhe, Germany) according to the instruction of the manufacturer.

**Resuspension buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>17.5 g</td>
<td>(f.conc. 300 mM)</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>5.6 g</td>
<td>(f.conc. 50 mM)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>0.68 g</td>
<td>(f.conc. 10 mM)</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>ad 1000 ml</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

All ingredients were dissolved in 250 ml water and subsequently adjusted to 500 ml. The pH was adjusted to 8.0.

**Wash buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>17.5 g</td>
<td>(f.conc. 300 mM)</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>5.6 g</td>
<td>(f.conc. 50 mM)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>3.4 g</td>
<td>(f.conc. 50 mM)</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>ad 1000 ml</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

All ingredients were dissolved in 250 ml water and subsequently adjusted to 500 ml. The pH was adjusted to 8.0.
Materials and Methods

**Elution Buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>17.5 g</td>
<td>(f.conc. 300 mM)</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>5.6 g</td>
<td>(f.conc. 50 mM)</td>
</tr>
<tr>
<td>Imidazole</td>
<td>17.0 g</td>
<td>(f.conc. 250 mM)</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>ad 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

Total volume 1000 ml

All ingredients were dissolved in 250 ml water and subsequently adjusted to 500 ml. The pH was adjusted to 8.0.

**SDS-Polyacrylamide gel electrophoresis**

For protein analysis SDS-polyacrylamide gels were casted of 10-12.5% acrylamide. The gels were casted and run with the Mini-protean® Tetra Cell system (Bio-Rad Laboratories GmbH, München, Germany) according to the manufactures instructions. Protein samples were prepared by mixing them 1:5 with SDS-PAGE loading dye. As a size marker the Dual Color Precision Plus Protein marker (Bio-Rad Laboratories GmbH, München, Germany) was used. The gels were run with Laemmlli SDS electrophoresis buffer 10 min at 150V and 45 min at 200V. After the run protein were overnight stained with colloidal Coomassie.
Chapter 2

**SDS-polyacrylamide resolving gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>10%</th>
<th>12.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution 30%</td>
<td>33.3 ml</td>
<td>41.7 ml</td>
</tr>
<tr>
<td>Resolving gel buffer solution 4X stock</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>SDS solution 10%</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>40.2 ml</td>
<td>31.8 ml</td>
</tr>
<tr>
<td>Ammonium persulfate solution 10%</td>
<td>500 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>33 µl</td>
<td>33 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>100 ml</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The acrylamide solution, the resolving gel buffer solution, the SDS solution and the demineralised water were mixed together. Just prior to polymerisation the ammonium persulfate and TEMED were added.

**SDS-polyacrylamide stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution 30%</td>
<td>700 µl</td>
</tr>
<tr>
<td>Stacking gel buffer solution 4X stock</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>SDS solution 10%</td>
<td>50 µl</td>
</tr>
<tr>
<td>Ammonium persulfate solution 10%</td>
<td>25 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

**Acrylamide solution 30%**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>300 g</td>
</tr>
<tr>
<td>N,N’-methylenebisacrylamide</td>
<td>8 g</td>
</tr>
<tr>
<td>Demineralised water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Materials and Methods

Resolving gel buffer solution 4x stock

Tris 60.6 g (f.conc. 1.5 M)

Demineralized water 850 ml

Total volume 1000 ml

First the Tris was dissolved in 750 ml water. The pH was adjusted to 6.8 and the remaining water was added. The solution was filtered through a 0.45-μm filter.

Stacking gel buffer solution 4x stock

Tris 181.7 g (f.conc. 1.5 M)

Demineralized water 850 ml

Total volume 1000 ml

First the Tris was dissolved in 750 ml water. The pH was adjusted to 8.8 and the remaining water was added. The solution was filtered through a 0.45-μm filter.

SDS-PAGE loading dye 6x

Tris 4.5 g (f.conc. 375 mM)

SDS 9.0 g (f.conc. 9% (w/v))

Glycerine 63 g (f.conc. 50% (v/v))

Bromophenol blue 30 mg (f.conc. 0.03% (w/v))

Demineralized water ad 100 ml

Total volume 100 ml

Laemlli SDS electrophoresis buffer 10X stock

Tris 303.0 g (f.conc. 250 mM)

Glycine 1441.0 g (f.conc. 1.92 M)

SDS (FW 288.38) 100.0 g (f.conc. 1% (w/v))
Colloidal Coomassie staining of SDS-PAGE gels

Colloidal Coomassie Blue G-250 working solution was prepared fresh. The volume needed depended on the box that was used for staining, but between 100-400ml. The gels were transferred the Colloidal Coomassie Blue G-250 working solution. Gels were stained between overnight and several days until spots appeared under gently shaking conditions. The gel was washed for 15 min. with demineralised water.

Colloidal Coomassie Blue G-250 stock solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulphate</td>
<td>50 g</td>
<td>(f.conc. 10% (w/v))</td>
</tr>
<tr>
<td>Phosphoric acid 85% (w/w) in water</td>
<td>6.0 ml</td>
<td>(f.conc. 1% (w/w))</td>
</tr>
<tr>
<td>Coomassie Blue G-250 5% solution</td>
<td>0.5 g</td>
<td>(f.conc. 0.1% (w/v))</td>
</tr>
<tr>
<td>Demineralised water ad</td>
<td>500 ml</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>500 ml</td>
<td></td>
</tr>
</tbody>
</table>

Colloidal Coomassie Blue G-250 working solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloidal Coomassie Blue G-250 dye stock solution</td>
<td>400 g</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>79 g</td>
<td>(99.8 ml)</td>
</tr>
<tr>
<td>Total volume</td>
<td>500 ml</td>
<td></td>
</tr>
</tbody>
</table>
Western Blotting

Before electrophoresis, samples were suspended in Laemmli sample buffer, boiled for 5 min at 95 °C and loaded onto an SDS-12% polyacrylamide gel and separated for 10 min at 100 V and 45 min at 200V followed by electrophoretic protein transfer at 150mA for 15 min and subsequently 300mA for 30 min to a PVDF membrane (Bio-Rad Laboratories GmbH, München, Germany). Polyclonal antibodies were diluted in TBS in appropriate ratio typically 1: 10.000 with 3% (w/v) bovine serum albumin (BSA, Carl Roth, Karlsruhe, Germany). The PVDF membrane was incubated with TBS for 2x 10 minutes. Thereafter the PVDF membrane was blocked with a 3% BSA solution for 1 hour followed by 2x 10 minutes washing with TBS-Tween and 1x 10 minutes with TBS. After that the PVDF membrane was incubated for 1 hour with the primary antibody. Subsequently the membrane was washed with 2x 10 minutes TBS-Tween and 1x 10 minutes TBS. Next the membrane was incubated with the secondary antibody for 1 hour. Anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad Laboratories GmbH, München, Germany) at a dilution of 1 : 5000 in TBS supplemented with 10% low-fat skim milk. The PVDF membrane was subsequently washed for 4x 10 minutes with TBS-Tween. The blot was developed with the ECL kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and luminescence was detected with a Stella bio imager (Raytest, Straubenhardt, Germany).

2D-gel electrophoresis secretome analysis

2D-gel electrophoresis

Three independently grown cultures of each strain P.aeruginosa PAO1 wildtype, ΔctpA and Δprc were pooled, centrifuged (6000 RCF, 4 °C), the supernatant was decanted, filtered through a 0.22 μm sterile filter (PVDF) and kept on ice during the experiment. Proteins were precipitated by adding 15 ml of 40% trichloroacetic acid (Carl Roth, Karlsruhe, Germany) in acetone to 15 ml supernatant and incubated at -20 °C for 2 h and subsequently centrifuged (18,000 RCF, 4 °C). The supernatant was discarded and the pellet washed with cold aceton ( -20 °C) three times and left to dry at the air. The protein pellet was dissolved in 750 μl rehydration buffer for isoelectric focusing and 250 μl were applied on a Immobiline Dry strip (pH-3-11 NL 13 cm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the
manufactures instructions and run on a Ettan IPGphor 3 IEF System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The second dimension was performed using a 12.5% SDS-polyacrylamide gel on a SE 600 Ruby gel electrophoresis system and stained with Coomassie Brilliant Blue according to the manufactures instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Protein spot intensities were measured with a Stella Bio Imager (Raytest, Straubenhardt, Germany) and analyzed with Delta 2D software (Decodon, Greifswald, Germany). Experiments were repeated four times.

**Rehydration buffer for isoelectric focusing**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
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<td>Urea</td>
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</tr>
<tr>
<td>Thiourea</td>
<td>3.8 g</td>
</tr>
<tr>
<td>CHAPS</td>
<td>0.5 g</td>
</tr>
<tr>
<td>IPG Buffer pH 3-11</td>
<td>500 µl</td>
</tr>
<tr>
<td>Bromophenol blue solution 1%</td>
<td>50 µl</td>
</tr>
<tr>
<td>Demineralized water</td>
<td>13.5 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

**MALDI-TOF peptide finger printing**

Protein spot were picked with a plastic Pasteur pipette and decolorized by adding 350 µl of a solution containing 30% (v/v) acetonitrile and 0.1 M ammonium bicarbonate. The solution was gently swirled for 10 minutes. The supernatant was removed and decolorized for a second time with the same solution for 10 minutes. After removing the supernatant the gel piece was dried for 10 minutes in a vacuum centrifuge. The gel pieces were rehydrated with 1 µL of a 10 ng/µL Trypsine (in Tris-HCl, pH 8.8) and the proteins were digested for 30 minutes at ambient temperature. Subsequently 4 µl of a 3 mM Tris-HCl buffer (pH 8.8) was added and incubated overnight at ambient temperature. The peptides were eluted by adding 6 µl water to the gel piece and incubated for 15 minutes. Added subsequently 5 µl of a solution containing 30% (v/v) acetonitrile and 0.1% (v/v) trifluoracid and incubated for 10 minutes. The solution containing the peptides were applied on a chip according to the manufactures instructions. The spot were identified by MALDI-TOF finger printing on a Bruker Ultraflex III (Bruker, Bremen, Germany).
Materials and Methods

Enzyme specific assays of supernatant

Cultures were grown of each strain as mentioned above for 16 hours. Supernatant was obtained by centrifuging the culture at 6,000 g for 30 min. at 4 °C and subsequent filtered through a PVDF 0.22 μm filter. During the experiment the supernatant was kept on ice.

Pseudolysin activity assay
Supernatant of three cultures were combined of each strain. 125 µl of the supernatant was mixed with 1 ml assay buffer. Assay buffer contained 10 mg Elastin Congo Red (Sigma-Aldrich, Saint Louis, USA) in 1 ml 10 mM Tris-HCl (pH 8.0). The reaction mixture was incubated for 3 hours at 37 °C and subsequently centrifuged at 10,000g for 15 minutes. The supernatant was decanted and absorbance was measured with a UV-Spectrophotometer at 490 nm. This assay was modified according to Caballero (Caballero et al., 2001). The assay was repeated 3 times.

Staphylolysin activity assay
Staphylococcus aureus (laboratory strain) was overnight grown in Luria-Bertani (LB) broth at 37 °C, on an agitating shaker at 150 r.p.m. The culture was centrifuged at 10,000 g and the supernatant discarded. The pellet was resuspended in 25 mM diethanolamine (pH 9.5). The suspension was heated at 100 °C for 10 minutes. The suspension was diluted with the diethanolamine buffer to a OD_{600} of 1.0. The assay was repeated 3 times.
Supernatant of three cultures were combined of each test strain. 40 µl supernatant was added to 1 ml of the Staphylococcus aureus suspension and incubated at 37 °C. Lysis was observed by measuring the OD_{600} every 1 minute during 1 hour. The assay was repeated 3 times.

Protease IV activity
The assay was modified according a method of Traidej, 2003 (Traidej et al., 2003). Supernatant of three cultures were combined of each strain. 10 µl of supernatant was added to 20 µl assay buffer. The assay buffer consisted of 3.15 mM Chromozym PL, 50 mM Tris, 150 mM Sodium chloride and 16 mM Ethylenediaminetetraacetic acid to inhibit the metallo-proteases Elastase
A and B, solved in water (pH 8.0). The mixture was incubated for 30 minutes at 37 °C. The absorbance was measured at 410 nm. The assay was repeated 3 times.

**Amino-peptidase PepB**

The activity of Amino-peptidase PepB was measured after a method of Kessler (2001). Supernatant of three cultures were combined of each strain. Assay buffer consisted of 1.2 mM L-Leucine-4-nitroanilide, 1 mM Calcium chloride and 50 mM Tris, solved in water, pH adjusted to 8.5. 20 μl of supernatant was added to 180 μl assay buffer. The mixture was incubated for 30 minutes at 37 °C. The absorbance was measured at 405 nm. The assay was repeated 3 times.

**Alkaline protease activity**

Alkaline protease activity was measured by adding 20 μl supernatant to 180 μl assay buffer. Assay buffer consisted of 1 mM Z-Arg-Arg-pNA.2HCl and 5 mM tris dissolved in water, pH adjusted to 7.8. The reaction mixture was incubated for 1 h at 30°C and subsequently measured at an absorbance of 410 nm. The assay was repeated 3 times.

**Localization of CtpA in P. aeruginosa (Hoge et al., 2011)**

**Cloning of pCtpA-lac**

Genomic DNA was isolated from P. aeruginosa PAO1. The ORF of PA5134 harboring the ctpA gene was amplified with Fw-ctpA-lac and Rv- ctpA-lac without the native promoter but maintaining the ribosome-binding site and was cloned into pBBR1-MCS1 by EcoRV to produce the pCtpA-lac. The pBBR1-MCS1 is an vector with chloramphenicol resistance gene and a lac-promotor which is constitutently transcribed in P.aeruginosa. The pCtpA-lac vector was transformed to E.coli DH5α cells. Clones were screening by Pvul restriction in order to identify ctpA insertion into the vector. The pCtpA-lac vector was transformed to E.coli S71.1 and transferred by biparental filter matings to P.aeruginosa PAO1.

**Fractionation of P. aeruginosa cells**
In order to detect the constitutently transcribed CtpA the *P. aeruginosa* strain harboring the pCtpA-lac vector was grown and cells were fractionized according to a slightly modified protocol as described by (Tielker et al., 2005). Briefly, the strain was grown to an OD$_{580}$ nm of 2 in 4 h. An 8-mL aliquot of culture was centrifuged for 10 min at 8,000 g. The supernatant was decanted and filtered through a 0.22-mm sterile PVDF membrane filter (Carl Roth, Karlsruhe, Germany). Proteins were precipitated by adding 1mL cold 40% (w/v) trichloroacetic acid in acetone (-20 °C) to 1mL supernatant and keeping at -20 °C. After 4 h, the mixture was centrifuged for 30 min at 20,000 g and 4 °C. The protein precipitate was washed twice with 100 mL ice cold 80% v/v acetone in water and dissolved in SDS-PAGE loading dye, comprising the extracellular fraction. For the whole-cell fraction, bacterial cells, harvested after centrifuging for 10 min at 8,000 g, were suspended in 100mM Tris-HCl (pH 8.0) and adjusted to OD$_{580}$ nm of 10. Spheroblasts were generated as follows: bacterial cells were carefully suspended in 100mM Tris-HCl (pH 8.0) with 20% w/v sucrose, adjusted to OD$_{580}$ nm of 10. After addition of the same volume of 100mM Tris-HCl with 5mM EDTA and 20 mg egg lysozyme, the sample was incubated for 30 min at room temperature. Spheroblasts were collected by centrifugation at 10,000 g for 20 min and the removed supernatant was used as the periplasmic fraction. The spheroblasts were disrupted by sonication (Sonifier W250, Branson) after the addition of the same volume of 100 mM Tris-HCl (pH 8.0). After centrifugation for 10 min at 5000 g to remove undisrupted cells and cell debris, the total membrane fraction was collected by centrifugation for 1 h at 13,000 g and the supernatant was used as the cytoplasmic fraction. An amount equivalent to an OD$_{580}$ nm of 0.5 of each fraction was used for Western blotting, except that for the extracellular fraction an amount of OD$_{580}$ nm of 2.5 was used.

**CtpA polyclonal antibodies**

CtpA polyclonal peptide-specific antiserum was generated against two synthetic synthesized peptides by immunization and boosting of a rabbit. The epitopes (H2N-CQIDGKPTKGQSMTEA-CONH2 and H2N-CGKRAAPSERPQDSDY-CONH2) were designed based of the deduced protein sequence of CtpA, synthesized and conjugated to keyhole limpet haemocyanin carrier proteins by the manufacturer (Eurogentec, Liège, Belgium). Polyclonal antibodies raised against exotoxin A from *P. aeruginosa* in a rabbit were purchased from Sigma. The generation of DsbA rabbit polyclonal antibodies were described elsewhere (Urban et al., 2001).
Western blotting of CtpA

The different subcellular fractions were loaded on an SDS-PAGE gel with PVDF membrane. Western blotting as performed as described above. The dilution used of the primary antibody of CtpA was 1:1000. As controls of the fractionation also western blots were performed with polyclonal antibodies of exotoxin A and DsbA in dilution of 1 : 5000 and 1 : 10 000, respectively.

Infection Models

Arabidopsis thaliana leaf infection assay

The assay was performed according to the method of Starkey & Rahme (Starkey and Rahme, 2009). Arabidopsis seeds were let to incubate on 0.1% Phytoblend agar, covered with foil and incubated at 41 °C. After 2 to 5 days the seeds were sown in potting soil and let to germinate at 20 °C with 50-70% humidity. The plants were grown with 12 hours of light per day. After germination the plants were grown for 3-4 weeks.

P. aeruginosa strains were grown as mentioned above. The cultures were centrifuged at 6,000g for 10 minutes and the supernatant was discarded. The pellet was washed with 1 ml of 10 mM magnesium sulphate. Subsequently the pellet was resuspended in 1 ml of 10 mM Magnesium sulphate and diluted to an O.D.\textsubscript{580} of 0.002 with the same solvent.

4 Plants were infected per strain, 4 leafs per plant. The leafs were infected by injecting each leaf with a 1 ml syringe until the leaf was saturated. Plants were incubated for 4 days at 30 °C with a humidity between 70-80% and a 12 hour per day light regime.

To monitor the infection, CFU growth was measured in the leafs. Therefore plant leaf samples were collected by taking 2 sample for each of the 4 leaf per strain. Samples were taken with a no. 3 cork borer. The two leaf discs from one leaf were washed with 1 ml 10 mM Magnesium sulphate. Subsequently the leaf discs were grind with a plastic pestle in 300 μl 10 mM Magnesium sulphate. After grinding to homogenous 700 μl of 10 mM magnesium sulphate was added. The sample suspension was diluted in 10 folds, four times. 20 μl of each dilution
was plated out on a LB agar plate and incubated overnight at 37°C. The next day colonies were counted and calculated as CFU per ml sample suspension.
Drosophila melanogaster infection model – general

Drosophila melanogaster Canton-S flies were grown on fly food at 25°C in fly incubators with high humidity (60–75%) and a 12-hour light/dark cycle. Fly food consisted of 1% (wt/vol) agar, 0.6% (wt/vol) sucrose, 3% (wt/vol) yeast, 4.4% (wt/vol) cornmeal supplemented with 0.36% (vol/vol) propionic acid and 0.11% (wt/vol) Tegosept®. For the experiment flies of 5-7 day old were used. Flies were anesthetized by using a CO₂ flow pad and only adult male flies were selected for the experiment.

Drosophila melanogaster infection model – feeding assay

P.aeruginosa strains were grown as mentioned above. The bacterial suspensions were centrifuged for 10 minutes at 6,000g. The supernatant was discarded and the pellet washed with a sterile 5% sucrose solution and subsequently resuspended in 175 μl of the same solution.

For the feeding assay 2x 15 flies per strain (wild type, ∆ctpA and ∆prc) were used. Flies were starved for 5 hours without water or feed. Afterwards flies were grown in fly containers with cotton, 2 ml of 5% sucrose and 175 μl bacterial strain. Flies were grown at 25 °C and monitored for mortality daily for 14 days. The experiment was repeated 2x (total 60 flies per strain).

Drosophila melanogaster infection model – needle pricking

P.aeruginosa strains were grown as mentioned above. 1 ml of the bacterial suspension was centrifuged for 2 minutes at 11,000g. The supernatant was discarded and the pellet washed with 1 ml 10 mM Magnesium sulphate. The pellet was centrifuged again and resuspended in 1 ml of 10 mM Magnesium sulphate. The bacterial suspension was diluted to an bacterial density of OD₅₈₀ 0.03 which corresponds about 3-5 x10⁷ cells per ml.

For the needle pricking assay 2x20 flies per strain were used. Pricking of the flies was executed with a tungsten needle which was sterilized by dipping in ethanol before dipping the tip in the bacterial suspension. The flies were pricked by inserting the needle parallel to the anteroposterior body axis in the dorsolateral abdominal cuticle close to the junction between the thorax and the abdomen. This procedure infects the fly with approximately 100 bacterial
Materials and Methods

cells. Flies were grown in fly food at 25 °C and mortality was monitored for 48 hours. The experiment was repeated 2x (total 80 flies per strain).

Statistical analysis of *D.melanogaster* survival kinetics was done using the Kaplan–Meier survival estimate with the SPSS software package (version 20, SPSS Statistics).

*Caenorhabditis elegans* infection model-slow killing assay
*C. elegans* Bristol N2 strain nematodes were generally cultured on nematode growth medium (NGM) in Petri-dishes and propagated at 23°C. The NGM medium consisted of 0.3% (wt/vol) sodium chloride, 1.7 (wt/vol) agar-agar, 0.25% (wt/vol) peptone, 1 μM calcium chloride, 5 μg/ml cholesterol, 1 μM magnesium sulplhate and 25 μM potassium phosphate in water (Brenner, 1974). *C. elegans* was fed on *Escherichia coli* OP50. *E. coli* OP50 is a uracil autotrophic organism whose growth is limited on NGM plates. *E. coli* OP50 was grown in LB-medium overnight at 37°C. After seeding NGM plates with *E.coli* OP50 bacteria are left to grown overnight at room temperature. For the assay nematodes were synchronized by subsequent hypochlorite bleaching, followed by hatching overnight and cultured on plates with NGM which were seeded with *E. coli* OP50.

*C.elegans* slow-killing assay was performed after a modified procedure from Tan & Ausubel (Tan and Ausubel, 2000). *P.aeruginosa* strains were grown as mentioned above. The cultures were diluted 100-fold in LB before using in the assay. 80 μl of the diluted *P.aeruginosa* cultures were spread on NMG-plates. Plates were subsequent grown for 24 hours at 37 °C. After cooling down of the plates, 50 larval stage 4 (L4) nematodes were applied onto the *P.aeruginosa* lawn. The plates with nematodes and *P.aeruginosa* strains were incubated at 24 °C. Mortality was monitored every 12 hours for 7 days. Nematodes were regarded death if they didn’t move onto tapping of the Petri-dish. Nematodes that died because of dehydration as a rest of crawling against the slides of Petri-dish were excluded.

Statistical analysis of *C.elegans* survival kinetics was done using the Kaplan–Meier survival estimate with the SPSS software package (version 20, SPSS Statistics).
**Galleria mellonella** infection model - injection assay

**Galleria mellonella** infection assay was performed modified according to Jander et al. (2000). *P. aeruginosa* strains were grown as mentioned above in triplicates to an OD_{580nm} of 0.03 in 20 ml LB broth at 37°C. 500 μl bacterial suspension was centrifuged for 2 min. at 10,000g. The pellet was suspended in sterile 10 mM MgSO₄, the OD_{580} was measured and adjusted to 0.05 with the same solution. 10 μl of 0.05 OD_{580} contains about 10⁶ CFU. Dilutions in 10 mM MgSO₄ were made to obtain solutions with bacterial concentrations of 10⁶, 10⁴, 10², 10¹, 10⁰ and 10⁻¹ CFU/10 μl. The triplicate dilutions were combined afterwards. 10 μl of each dilution was injected with a HumaPen LUXURA HD® insulin injection pen (Eli Lilly, Giessen, Germany) into *G. mellonella* caterpillars in the final larval stage. The larvae were stored in the dark before using. 10 larvae were injected per dilution of each strain and incubated for 3 days at 24 °C. After day 3 the larvae were scored death or alive. The larvae were scored death if no movement was observed after touching. Exact bacterial concentration of the dilutions was determined by plating out on LB-agar (8 fold), incubate for 2 days at 24 °C and count the CFU. The results of three independent experiments were combined.

LD_{50} values (Lethal Dose for 50% of subjects) were estimated with the SPSS software package (version 20, SPSS Statistics). The LD_{50} values were estimated by Probit analysis.

**Galleria mellonella** infection model – supernatant injection assay

*P. aeruginosa* cultures were grown as described above. Sterile supernatant was obtained by centrifugation (6000g, 4 °C), decanting the supernatant and finally filtering through a 0.22 μm sterile filter (PVDF). The supernatant was kept on ice during the experiment. The sterility of the supernatants were proofed by growing 100 μl in 20 ml liquid LB medium and incubate for 2 days at 37 °C. The supernatant was regarded valid if no growth was observed. The supernatant was injected with a HumaPen LUXURA HD insulin injection pen (Eli Lilly, Giessen, Germany) into *G. mellonella* caterpillars in the final larval stage. The larvae were stored in the dark before using. 10 larvae were injected with 20 μl supernatant of each strain and incubated for 6 days at 37 °C. Mortally was monitored every 24 hours for 6 days. The larvae were scored death if no movement was observed after touching. As a control 5 larvae were injected at the same time with 20 μl LB medium. The experiment was repeated 3 times.

Statistical analysis of larvae survival kinetics was done using the Kaplan–Meier survival estimate with the SPSS software package (version 20, SPSS Statistics).
Materials and Methods

Cloning and overexpression of CTPs

Genomic DNA was isolated from *P. aeruginosa* PAO1 wild type and *Escherichia coli* K12-MG1655. ORFs PA5134 and PA3257 encoding the CTPs CtpA and Prc were amplified from the genomic DNA with primers Fw-ctpA19b and Rv-ctpA19b (PA5134) and respectively Fw-prc19b and Rv-prc19b (PA3257). ORF b1830 of *E. coli* encoding Prc was amplified from the genomic DNA with primers Fw-prcEc19b and Rv-prcEc19b. These three ORFs were cloned into the plasmid pET-19b using the restriction enzymes NdeI and XhoI. Mutagenesis of CtpA and Prc was performed by overlap extension PCR. CtpA serine 302 was changed to alanine by amplification of fragment 1 (Fw-ctpASA and ctpA302A) and 2 (ctpA302A and Rv-ctpASA) with pET-19b-CtpA as template and subsequent amplification with primers Fw-ctpASA and Rv-ctpASA with fragment 1 and 2 as template followed by insertion in pET-19b using NdeI and XhoI. The three other variants CtpA-K327A, Prc-S479A and Prc-K504A were constructed in a similar way. CtpA lysine 327 was exchanged for alanine by amplification of fragments 3 (Fw-ctpAKA and ctpAK327A) and 4 (ctpAK327A and Rv-ctpAKA) with pET-19b-CtpA as template; Prc serine 479 was exchanged for alanine via amplification of fragments 5 (Fw-prcSA and prcS479A) and 6 (prcS479A and Rv-prcSA) with pET19-Prc as template; Prc lysine 504 was exchanged for alanine via amplification of fragments 7 (Fw-prcKA and prcK504A) and 8 (prcK504A and Rv-prcKA) with pET-19b-Prc as template. Plasmids were transformed to in *E. coli* DH5α. The protease were overexpression using both the *in vitro* as *in vivo* overexpression systems.

**In vivo overexpression**

The overexpression of proteases in vivo was examined in *E.coli* JM109(DE3) and BL21(DE3). The overexpression experiments were started at 37 °C und subsequently lowered to 25 °C. Overexpression was also executed with use of auto induction medium. Cultures were grown for 16 hours at 37 °C. The proteases were purified with the 6-His-tag as described above.

**In vitro overexpression**
All proteases were also expressed using about 2 µg of vector DNA by *In vitro* overexpression as described above.

**β-casein proteolytic assay**

CTP protease were assayed on β-casein activity. The assay was executed as follows.

**β-Casein assay**

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</tr>
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<tr>
<td>β-casein assay buffer</td>
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<td>µl</td>
</tr>
<tr>
<td>(f.conc. 4 mM)</td>
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<td></td>
</tr>
<tr>
<td>Assay buffer</td>
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</tr>
<tr>
<td>Total volume</td>
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</tbody>
</table>

The assay was incubated at 37 °C for 16 hours. After that a 5 µl sample of the assay was analysed by SDS-PAGE as described above.

**Assay buffer**

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<tr>
<td>KCl</td>
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<td>g</td>
<td>(f.conc. 200 mM)</td>
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<td>ml</td>
</tr>
<tr>
<td>Total volume</td>
<td></td>
<td>100</td>
<td>ml</td>
</tr>
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</table>

The pH was adjusted to 8.0.

**β-Casein assay buffer**

<table>
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<tr>
<th>Component</th>
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<th>Unit</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-casein</td>
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<td>Total volume</td>
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</table>

The pH was adjusted to 8.0.

**Enzyme activity of synthetic PBP3-peptides, D1-peptides and tmRNA peptide**
The cleavage ability of CtpA, Prc from *P. aeruginosa* and Prc from *E. coli* were tested in an assay with short C-terminal peptides. The sequence of the peptides are listed below.

**PBP3-*P. aeruginosa***

FITC-Acp-MAGALRLMNVPDNLPTATEQQVNAAPAKGGRG

**PBP3A-*P. aeruginosa***

FITC-Acp-MAGSLRALAIIPDNLQDSPAVADRQHHG

**PBP3-*E. coli***

FITC-Acp-TMNIEXPDLTGDKNFVINVGEGRGTGRS

**tmRNA-peptide *P. aeruginosa***

FITC-Acp-AARAAK-Ahx-Ahx-DNYALAA

**D1-*S. oleracea***

FITC-Acp-VMHERNAHFPLDLAAIEAPSTNG

**D1-*S. obliquus***

FITC-Acp-VMHERNAHFPLDLASVEAPSVNA

The peptides correspond for a small C-terminal part of Penicillin Binding Proteins from *P. aeruginosa* (PBP3 and PBP3A) and from *E. coli* (PBP3) and D1 precursor proteins from *S. oleracea* and *S. obliquus* and there is one peptide that mimics the tmRNA tagging peptide of *P. aeruginosa*. The peptides were purchased from Biomatik (Wilmington, USA) with a purity of > 95%. A Fluorescein isothiocyanate (FITC) tag was labeled at the N-terminally peptides with the use of 6-aminohexanoic acid (Ahx) as linker. The C-terminal had a free carboxy acid group. Cleavage assays were conducted in 100 µl 50 mM ammonium carbonate, pH 8.0 with 200 nM protease and 15 µM substrate peptide at 37°C for 16 hours. The proteases were inactivated
by incubation at 100 °C for 5 minutes and subsequently cooled on ice. The samples were analyzed by HPLC and LC-MS.

**HPLC analysis and mass spectrometry**

A 250 x 4 mm BioBasic-18 column, 5 µm particle size, 300 Å (Thermo Fisher Scientific GmbH, Dreieich, Germany), equipped with a pre-column (10 x 4 mm) filled with the same material, was used with a gradient of acetonitrile (A) with 0.09% trifluoroacetic (TFA) or in the case of mass spectrometry with formic acid (FA) and water (B) with 0.1% TFA respectively FA.

All LC measurements were performed on LC-10Ai series (Shimadzu, Japan) installed with LabSolution/LC Solution program version 1.22SP1. The system was equipped with an RF-10AXL fluorescence detector. The column oven temperature was maintained at 30°C, the flow rate was set to 1 mL/min and the injected volume was set to 10 µL. The fluorescence detection was carried out by 494 and 518 nm for the excitation and emission wavelengths, respectively. The linear gradient started at initial conditions from 5:95 (A:B), followed by further 25 min to 57:43 (A:B), from 25 to 28 min to 65:35 (A:B), 100:0 (A:B) in 0,1 min, 100:0 (A:B) from 28,1 to 31 min, in 0,1 min finally returned to initial conditions and re-equilibration for 5 min.

The LC-MS experiments were carried out on an Agilent 1100 series with a binary pump system (Agilent Technologies, Waldbronn, Germany), coupled with a 4000QTrap™ linear ion trap mass spectrometer (Applied Biosystem/MDS SCIEX, Framingham, USA) equipped with a Turbolon spray source and controlled by Analyst® 1.5. Mass reconstruction of peptides was done by Bioanalyst® (Applied Biosystem/MDS SCIEX, Framingham, USA).

The separation was achieved on the column described before. The gradient elution was done with deionized water with 0,1% FA (solvent A) and acetonitrile with 0,1% FA (solvent B) at a constant flow rate of 1 mL/min and a split ration of 1:1 before ion source were used. The gradient profile was 5% B for 2 min, from 2 to 25 min a linear increase from 5% B to 57% B, a linear increase to 95% B at 26 min with a step for 6 min and a return to 5% B at 33 min and 7 min for re-equilibration. The injection volume was 10 µL.

The mass spectrometer was operated in the positive Enhanced MS (EMS) mode scanning from 400 – 1800 Da with a dynamic LIT fill time amd a scan rate of 4000 Da/s. The parameters used were optimized first performing a Flow Injection Analysis (FIA) with the peptides as standards
Materials and Methods

amd led to the following parameter settings: IS 4500 V, Declustering Potential (DP) 210 V, Curtain Gas (N2) 10 arbitrary units (au), Source Temperature 400°C, Nebulizer Gas (N2) 50 au and Heater Gas (N2) 20 au. CE and Q3-Entry barrier were set to 5 V and 8 V, respectively.

High Throughput assay development

EDANS/DABSYL pD1 substrate

DABCYL-VMHERNAHFPLDLA SVE(EDANS)APSVNA

The EDANS/DABSYL pD1 substrate was dissolved in 25% (v/v) acetonitrile in assay buffer. The solution was diluted with assay buffer until the final concentration substrate were 200 μM and 5% acetonitrile (v/v). Enzyme kinetics were determined by diluting the substrate solution in 9 different concentration ranging from 0; 0,5; 2,5; 5; 7,5; 10; 15; 30; 50 or 100 μM and 200 nM protease. The kinetics were measured over time with a Tecan Infinite M200 platereader (Tecan Group Ltd, Männedorf, Switzerland). The excitation wavelength was set at 335 nm and emission wavelength was 493 nm. The experiment were executed three times. Michaelis-Menten parameters were calculated by GraphPad Prism.

Inhibition measurements

The compounds Rhodanine dimer A and B tested for inhibition.

Rhodanine dimer A

2,6-Bis[5-[p-(dimethylamino)benzylidene]-4-oxo-2-thioxo-3-thiazolidinyl]- hexanoic acid

Rhodanine dimer B

2,6-Bis[5-furanylidene-4-oxo-2-thioxo-3-thiazolidinyl]- hexanoic acid

For the assay the EDANS/DABSYL pD1 substrate was dissolved in assay buffer to a concentration of 15 μM and 200 nM protease. The inhibitors were dissolved in DMSO.
inhibitors were added to obtain different concentrations ranging from 0, 10, 25, 33, 45, 60, 85, 120, 225 μM. The assay contained maximal 1% DMSO. Enzyme kinetics were measured over time with a Tecan Infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). The excitation wavelength was set at 335 nm and emission wavelength was 493 nm. The experiment was executed three times. Inhibition parameters were calculated by GraphPad Prism.

$K_i$ values were calculated using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \left(\frac{S}{K_m}\right)}$$

**Software**

Survival curves and LD$_{50}$ values were determined with SPSS software package (version 20, SPSS Statistics, IBM, Armonk, New York). N-signal peptide prediction was performed by the SignalP software (Petersen et al., 2011). Cloning strategies and vector charts were designed by using the Clone Manager® software (version 7, Scientific and Educational Software, Cary, USA). Gene and protein sequences and information were obtained from the Pseudomonas Genome Database (www.pseudomonas.com; Winsor et al., 2011). Multiple alignments of protein sequences were performed with the T-coffee® software (www.t-coffee.org; Notredame et al.) and analyzed with the BioEdit® software (Ibis Biosciences, Carlsbad, USA). Homology searches were performed with the Basic Local Alignment Search Tool (BLAST®; http://blast.ncbi.nlm.nih.gov; Altschul et al., 1997)). 2D gel electrophoresis analysis was performed with the Delta 2D software (Decodon GmbH, Greifswald, Germany). Enzyme kinetics and inhibition parameters were calculated using the GraphPad Prism® 5 software (version 5, GraphPad Software Inc., La Jolla, USA).
References


"The first iterative pattern can be seen"
CHAPTER 3

CTPs: Carboxy-terminal processing proteases
Chapter summary

Carboxy-terminal proteases (CTPs) are endoproteases which preferably cleave proteins at their carboxy-terminus. CTPs are widespread within the kingdoms and can be found in eukaryotes such as Archaeplastida and in prokaryotes such as Bacteria and Archaeae.

CTPs are classified as protease family S41. In bacteria CTPs can be divided into three major subfamilies named CTP-1, CTP-3 and CtpB. Many bacterial genera contain one CTP copy in their genomes although the presence of multiple copies is not uncommon. This wide conservation of CTPs across the bacterial kingdom suggests an basal and important physiological function. The genus Pseudomonas harbors two paralogous CTP proteases belonging to the two different subfamilies of CTP-1 and CTP-3.

CTPs of Gramm negative bacteria seems to be localized in the periplasm and are not secreted in the external environment.

The first bacterial CTP that was identified and characterized was a protease from Escherichia coli named Tsp. The Tsp showed a preference of cleavage for a λ repressor variant #105 with a hydrophobic carboxy terminus.

CTPs have shown to be involved in a wide range of pleiotropic effects and are able to cleave a wide range of different bacterial substrates as shown in E.coli, Pseudomonas aeruginosa, Borellia burgdorfi, Burkholderia mallei, Brucella suis, Chlamydia trachomatis and Rhizobium leguminosarum.

CTPs play a role in the pathogenesis of bacteria as shown in mice infected with mutant strains of B.mallei and B.suis.
Carboxy-terminal processing proteases

History

Carboxy-terminal processing proteases are endoproteases which exist in eukaryotes such as Archaeplastida and in prokaryotes such as Bacteria and Archeae. Endoproteases are proteases or peptidases that hydrolyse covalent peptide bonds of proteins or peptides and by therefore cleave the chain. The name of protease, proteinase of peptidase is interchangeable. Carboxy- or C-terminal processing proteases (CTPs) cleave preferably at the C-terminus of their target peptides. The Tsp protease from Escherichia coli was the first bacterial CTP purified and characterized (Silber et al., 1992). Although earlier isolations of proteases may likely be the same protease: such as the isolation of a protease that cleavages oxidized Glutamine synthetase in E.coli by Roseman and Levine or that of Protease Re by Park and coworkers (Park et al., 1988; Roseman and Levine, 1987).

Silber and coworkers purified a protease and showed that it preferably cleavages a λ repressor variant #105 with a polar C-terminal amino-acid sequence WVAAA and doesn’t cut the wild type protein with the C-terminal amino-acid sequence RSEYE (Silber et al., 1992). They therefore named the protease; Tsp (Tail Specific Protease). Hara and Nagasawa published in two accompanying papers the involvement of Tsp in the processing of the Penicillin-binding-protein 3 (PBP3) and determined the cleavage site by sequencing the PBP3 variants of an inactive prc strain and wildtype (Hara et al., 1989; Nagasawa et al., 1989). The mature PBP3 seemed to being processed by involvement of the prc gene. The prc gene codes for the Tsp protease in E.coli. Prc stands for “Processing involving C-terminal cleavage” (Silber et al., 1992). The protease is therefore named either Tsp or Prc. In most scientific literature Prc is the prevailing name which I will use in this work.

C-terminal processing proteases are also present in Archaeplastida such as plants, algae and cyanobacteria. What Viridiplantae characterizes is the presence of chloroplasts which use light to make energy. An important subunit of photosystem II is the D1 protein. Marder and coworkers showed in Spirodelta oligorrhiza that the D1 is synthesized in the cell as a precursor protein and is processed at the C-terminal part before being installed in the active
photosystem II (Marder et al., 1984). The protease that was involved in this processing was identified and purified from *Scenedesmus obliquus* and spinach (*Spinacia oleracea*) and later cloned (Fujita et al., 1995; Inagaki et al., 1996). The protease was named CtpA after "Carboxyl-terminal processing protease A" as well as the gene *ctpA* (Anbudurai et al., 1994).

Another type of C-terminal processing proteases are also found in Archeae. In 2001 Tamura and coworkers isolated a typical protease from *Thermoplasma acidophilum* and named it Tricorn protease. This Tricorn proteases is the central part of a proteolytic system (Tamura et al., 1996). The protease is named after the Tricorn hat that has a typical triangular shape (Tamura, 2004).

**Genetic distribution and profile**

Based on the ever increasing available sequenced genomes and subsequent annotation by homology searches much can be said about the C-terminal processing proteases distribution in organisms. CTPs can be found in almost every kingdom such as Bacteria, Archa, Plants and animals. Until now there’s only one homolog found in a Protoza and non in Fungi or Viruses. Much condensed data about peptidases can be found in the MEROPS database of the Sanger Institute (www.merops.sanger.co.uk) [Rawlings, 2012].

Most genomes of bacteria harbor a CTP homologue which can be predominately divided in three subfamilies based on sequence alignments: C-terminal processing peptidase-1 (CTP-1), C-terminal processing peptidase-3 (CTP-3) and CtpB peptidase (CtpB). There are much more subfamilies known but contain until now only a few specific bacterial proteases. In some bacteria species CTP homologues couldn’t identified in the until now complete sequence genomes such as: *Corynebacterium spp.*, *Francisella spp.*, *Mycobacterium spp.* and *Streptococcus spp*. All subfamilies are based on the sequence of a holotype. The different subfamilies with holotypes and the domain architecture of their genes are depicted in figure 3.1.
CTPs: Carboxy-terminal processing protease

C-terminal processing peptidase-1 (CTP-1)  Holotype: Tsp (Escherichia coli)

C-terminal processing peptidase-3 (CTP-3)  Holotype: CtpA (Bartonella bacilliformis)

C-terminal processing peptidases B (CtpB)  Holotype: ctpB (Bacillus subtilis)

Figure 3.1: Schematic holotype gene domain structures of bacterial Carboxy-terminal processing proteases S41 subfamilies.
All CTPs contain a PDZ domain which is believed involved in the substrate (protein-protein) recognition of the proteases. The domain is followed by the peptidase domain which is involved in the hydrolysis of the peptide bond and therefore executes the cleavage. In subfamily CTP-1 an additional domain has been identified based on sequence alignment, with an until now unknown biological function. The domain is named in the PFAM database as C-terminal domain of tail specific protease (PFAM identifier: DUF3340) which have only be indentified in these particular proteases (Punta et al., 2012). Also subfamily CtpB has an additional domain named: Putative peptidoglycan binding domain (PFAM identifier: PF01471) which are thought to have a general peptidoglycan binding function and to be involved in bacterial cell wall degradation (Punta et al., 2012).

Furthermore CTP-1 and CTP-3 family genes mostly have also a signal peptide on the N-terminal of the protein. This signal peptide predominantly directs the proteases to the periplasm of Gram-negative bacteria (Hoge et al., 2011).

Plants and other Viridiplantae such as Algae have CTPs belonging to the family: C-terminal processing peptidase-2 (CTP-2). As can been seen in figure 3.1 the domain architecture of this family resembles bacterial family CTP-3 with a distinct PDZ domain and subsequent catalytic peptidase domain.
Table 3.1: overview of distribution and type of CTPs in miscellaneous pathogenic bacteria.

<table>
<thead>
<tr>
<th>Pathogenic bacterial species</th>
<th>Amount of CTP homologues</th>
<th>CTP subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlamydia trachomatis</strong></td>
<td>1</td>
<td>Ctp-1</td>
</tr>
<tr>
<td><strong>Chlamydia pneumoniae</strong></td>
<td>1</td>
<td>Ctp-1</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>1</td>
<td>Ctp-1</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong></td>
<td>2</td>
<td>Ctp-1</td>
</tr>
<tr>
<td><strong>Shigella dysenteriae</strong></td>
<td>3</td>
<td>Ctp-1</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>2</td>
<td>Ctp-1 (1x), Ctp-3 (1x)</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td>6</td>
<td>Ctp-1 (3x), unassigned (3x)</td>
</tr>
<tr>
<td><strong>Moraxella catarrhalis</strong></td>
<td>2</td>
<td>Ctp-1 (1x), unassigned (1x)</td>
</tr>
<tr>
<td><strong>Salmonella enterica</strong></td>
<td>16</td>
<td>Ctp-1 (7x), unassigned (9x)</td>
</tr>
<tr>
<td><strong>Vibrio cholerae</strong></td>
<td>2</td>
<td>Ctp-1 (1x), unassigned (1x)</td>
</tr>
<tr>
<td><strong>Yersinia pestis</strong></td>
<td>3</td>
<td>Ctp-1 (2x), unassigned (1x)</td>
</tr>
<tr>
<td><strong>Bartonella henselae</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Bordetella pertussis</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Campylobacter jejuni</strong></td>
<td>2</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Clostridium perfringens</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Coxiella burnetii</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Legionella pneumophila</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Neisseria gonorrhoeae</strong></td>
<td>1</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Treponema pallidum</strong></td>
<td>2</td>
<td>Ctp-3</td>
</tr>
<tr>
<td><strong>Burkholderia cenocepacia</strong></td>
<td>4</td>
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<tr>
<td><strong>Brucella abortus</strong></td>
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<td>Ctp-3 (2x), unassigned (1x)</td>
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<td><strong>Clostridium botulinum</strong></td>
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<td><strong>Clostridium difficile</strong></td>
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<td><strong>Clostridium tetani</strong></td>
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<td><strong>Neisseria meningitidis</strong></td>
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<tr>
<td><strong>Bacillus anthracis</strong></td>
<td>1</td>
<td>ctpB</td>
</tr>
<tr>
<td><strong>Bacillus cereus</strong></td>
<td>5</td>
<td>ctpB</td>
</tr>
<tr>
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<td>3</td>
<td>ctpB (3x)</td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong></td>
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<td>ctpB (3x)</td>
</tr>
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<td><strong>Enterococcus faecalis</strong></td>
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<td>ctpB (3x), unassigned (1x)</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
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<td>ctpB (1x), unassigned (2x)</td>
</tr>
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<td><strong>Borrelia burgdorferi</strong></td>
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<td>S41.013 CtpA peptidase (Borrelia-type)</td>
</tr>
<tr>
<td><strong>Corynebacterium diptheriae</strong></td>
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<td></td>
</tr>
<tr>
<td><strong>Ehrlichia chaffeensis</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Francisella tularensis</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Mycobacterium leprae</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Mycobacterium tuberculosis</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Mycoplasma pneumoniae</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pneumonia</strong></td>
<td>non</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pyogenes</strong></td>
<td>non</td>
<td></td>
</tr>
</tbody>
</table>
Sub cellular localization

Hara et al. and Silber et al proposed that Prc of *Escherichia coli* is localized in the periplasm based on indirect evidence (Hara et al., 1991; Silber et al., 1992). There is still doubt that CTP were not secreted extracellular. I proofed that CtpA of *Pseudomonas aeruginosa* was translocated over inner cellular membrane to the periplasm by sub cellular fractionation and subsequent western blotting and is not secreted to the external environment (chapter 7).

CTPs of the CTP-1 and 3 families which can be found most predominately in Gram-negative bacteria have a N-terminal signal peptide which directs the protein for translocation over the inner membrane. Subcellular localization of CTPs in Gram-positive bacteria have not yet investigated. Bioinformatic analysis of predicted CTPs of Gram-positive bacteria such as *Bacillus subtilis* and *Clostridium difficile* suggest N-terminal signal peptides that would translocate the CTPs across the cell membrane (Hoge et al., 2011).

The CTP-2 proteases in plants and algae are located within the thylakoid lumen. The CTP-2 proteases are being encode within the genomic DNA and contain a stromal-import sequence and a signal sequence for translocation across the thylakoid membrane into the thylakoid lumen (Satoh and Yamamoto, 2007).

Biological function

Much is unknown about the biological function of CTPs in bacteria. The wide occurrence of predicted CTP sequences in whole bacterial genomes suspects a basic physiological function. In the initial papers of Hara and coworkers they showed that a prc (CTP-1) mutant in *E.coli* showed thermosensitive growth on a salt free and ½ LB medium (low osmolarity) (Hara et al., 1991). The mutant strain was identified in the research involving Penicillin Binding Protein 3 of *E.coli*. This mutant showed an unprocessed PBP3 with was related to a non functional prc gene (Hara et al., 1989). Protein Binding Proteins-3 are involved in the septation process (Nguyen-Distèche et al., 1998).
Tadokoro and co-workers showed that Prc of *E.coli* interacts with lipoprotein NlpI which seems only be functional when the C-terminal is processed. NlpI may be involved in septum formation or in cell wall degradation during cell division (Ohara et al., 1999). Prc of *E.coli* is also involved in the processing of TonB and the Arc repressor. TonB is a periplasmic protein that is involved in the binding of outer membrane proteins (Keiler et al., 1995). Silber and co-workers showed that Prc from *E.coli* degrades the #105 protein a variant of the λ-suppressor protein (Silber, 1992). The #105 protein has a more hydrophobic carboxy-terminal (amino acids sequence: WVAAA) than the λ-suppressor (amino acids sequence: RSEYE). Whether Prc cleaves the #105 also in vivo is unclear. The #105 variation is at least being processed in vivo in inactivated prc mutants (Keiler et al., 1995). Mutants of prc *E.coli* showed leakage of periplasmic protein as the result of a leak outer membrane (Hara et al., 2002; Hara et al., 1991). Mutants of prc were more susceptible for antibiotics among them tetracycline, nalidixic acid, erythromycin, spectinomycin, norfloxacin, and novobiocin (Seoane et al., 1992). The prc (CTP-1) of *Pseudomonas aeruginosa* was shown to be involved in the regulation by promoting mucoidy formation.

Mucoidy isolates of *P.aeruginosa* overproduce exopolysaccharide alginate and is a cause of chronic respiratory infections at patients with cystic fibrosis (CF) (Reiling et al., 2005). The overproduction is caused by mutant forms of the mucA gene. It was suggested that Prc is involved in the degradation of the mutant MucA proteins. In another study Prc of *P.aeruginosa* have shown to activate algD expression after treatment with D-cycloserine suggested its involvement in response to cell wall stress (Wood et al., 2006).

In the Lyme disease spirochete *Boreillia burgdorferi* a CtpA (CTP-3) mutants strain showed pleiotropic effects in the secretion of different proteins (Ostberg et al., 2004). P13, an 13 kDa outer surface-exposed integral outer membrane protein with unknown function, was shown to be process at the C-terminus (Noppa et al., 2001). Just like BB0323, a putative membrane protein, which function is also unknown.

In *Burkholderia mallei* a CtpA (CTP-3) showed various cell morphologies and disintegrated cell envelopes (Bandara et al., 2008).

Another intracellular living pathogen is *Brucella suis*. Mitchel and coworkers showed that CtpA (CTP-3) from *Brucella suis* undergoes autolysis (Mitchell and Minnick, 1997). In another study
on *B. suis* Bandara and coworkers showed that the ΔctpA strain had an altered morphology in the form of spherical cells in stand of the native coccobacillus shape when grown on normal LB agar. The ΔctpA strain was unable to grow on salt free medium by losing cell integrity followed by lysis (Bandara et al., 2005).

The CtpA (CTP-3) of *Chlamydia trachomatis* was shown to be able of cleavage of the human p65/RelA protein. The p65/RelA protein is a transcription factor within the NF-κB pathway. The cleavage of the p65 abolished the immune activation. The authors suggest that by this mechanism the *C. trachomatis* is able to evade the host immune system and is able to survive intracellular (Lad et al., 2007).

Studies performed on *Rhizobium leguminosarum* showed that the ΔctpA mutant (CTP-3) was more sensitivity for desiccation (Gilbert et al., 2007). The CtpA defective mutant was also unable to grown on nutrient rich media. The mutant strain was also more sensitive for detergents such as sarcosyl and sodium deoxycholate. The bacteria of the mutant strain had also a spherical morphology where as the wild type strain is normally rod shaped (Gilbert et al., 2007).

**Pathogenesis**

*Burkholderia mallei* is an pathogen that establishes intracellular infections of the host. The ΔctpA *Burkholderia mallei* a CtpA (CTP-3) less able to grown in macrophages when compared to the wild time (Bandara et al., 2008). In this study Bandara and coworkers showed that the ΔctpA was less virulent in mice and showed an dose depending lethally after intraperitoneal infection. Mice infected with a non lethal dose of the ΔctpA strain produced specific serum immunoglobulins G and were partially protected against infection with *B. mallei* (Bandara et al., 2008). The authors suggested that the ΔctpA could be further developed as an live vaccine for *B. mallei* diseases such as Glanders.

The same group of Bandara also investigated a CtpA mutant of *Brucella suis*. The CtpA defective strain survived much less in macrophages as the wild type strain. This survival is essential of the intracellular pathogen. The mutant CtpA strains was also less pathogenic as
tested in mice. After nine weeks following an intraperitoneal infection, the mutant could not be detected in the spleen anymore as the wild type was recovered from the spleen. Also were mice infected with the ΔctpA strain well protected for the server pathogenic B. suis strains. The method was patented for the use as a Brucella suis vaccine at the USA patent office (Bandara et al., 2007).

**Human CTP homologue**

The human genomes also comprises a homologue of bacterial C-terminal processing proteases. The protein is named Interphotoreceptor retinoid-binding protein (IRBP). The IRBP is a 135 kDa big protein which is composed of multiple homologues repeats (4 modules) (Baer et al., 1998). The human IRBP gene structure is depicted in figure 3.2.

5 domains are identified in the gene (RBP3) coding the IRBP. Four domains are homologues to the catalytic domain of the bacterial CTP proteases. The IRBP protein is the major soluble constituent of the Interphotoreceptor Matrix in the human retina. The IRBP plays a role in the transport of retinoids between the rods and the retinal pigment epithelium (Baer et al., 1998). In the rhabdoms, 11-cis retinaldehyde (vitamine A aldehyde) is bound to rhodopsin. Rhodopsin with its chromophore, is a G-coupled protein, which catches light after which a secondary signal transduction occurs. The capture of light transforms the Rhodopsin to Metarhodospin and the 11-cis retinaldehyde to all-trans retinaldehyde (Gonzalez-Fernandez, 2003). IRBP is able to bind both 11-cis retinaldehyde and all-trans retinaldehyde which are by itself insoluble. The IRBP traffic the retinoids between the photoreceptors and the retinal pigment epithelium and protects them for isomerisation and oxidative degradation (Baer et al., 1998). Defects in the RBP3 gene have been associated Retinitis pigmentosa, a human visual inherited disease (Sato et al., 2013). The domains of the four modules in the RBP3 gene are similar to the S41 peptidase domain of the bacterial CTPs. Although based on sequence alignments no peptidase activity is predicted and the modules are therefore no proteases (Rawlings et al., 2012). The DUF3436 domain has an until now unknown function (PFAM identifier: DUF3436) (Punta et al., 2012).
Figure 3.2: Schematic gene domain structure of the human Interphotoreceptor retinoid-binding protein.
References


CTPs: Carboxy-terminal processing protease


"Its mass grows exponentially"
Proteases and their role in virulence of

*Pseudomonas aeruginosa*

This chapter is based on:

Chapter summary

Pathogenic bacteria need to interact with their host to establish an infection and to maintain it successfully afterwards. This requires to “understand” signals of the immune system and to “respond” appropriately when the host tries to defend himself. Part of the response of successful pathogens is secretion of so called virulence factors which manipulate or even destroy defense lines of the host. An important part of the arsenal of bacterial virulence factors are proteases. As proteins are one of the basic building blocks in nature, proteases can influence a broad range of biological functions. The opportunistic human pathogen *Pseudomonas aeruginosa* has an arsenal of impressively efficient proteases that helps establishing and maintaining an infection and thereby controlling and modifying the environment according to the needs of the bacterium.

One of those secreted virulence factors is elastase B, a metalloprotease, which has been shown to have a role in the pathogenesis of respiratory infections, especially in patients suffering from Cystic fibrosis. Elastase B degrades the human protein elastin, which is an important protein in lung tissue where it stabilizes the respiratory epithelium. Elastase A or “staphylolysin” is complementary to elastase B in weakening lung tissue by nicking the elastin. Interestingly, elastase A also plays a role in the defense of *Pseudomonas* against rivals in the fight for resources from which the enzyme received its name “Staphylolysin”, since it can degrade the cell wall of Gram-positive bacteria such as *Staphylococcus aureus*. A third virulence factor is the serine protease “Protease IV” which has been show to play an important role in keratitis during a corneal eye infection. Alkaline protease can inhibit the function of the cells of the immune system (phagocytes, NK cells, T cells), it inactivates several cytokines (IL-1, IL-2, IFN-γ, TNF), destroys immunoglobulins and inactivates several components of the complement system.

Understanding these mechanisms offers us the opportunity to develop next generation antibiotics which help to undermine the attack of pathogenic bacteria.
Proteases and their role in virulence of *Pseudomonas aeruginosa*

There are several well known bacterial proteases that interact with their hosts during a pathogenic infection. The highly lethal Anthrax Toxin of the pathogenic bacterium *Bacillus anthracis* consists for example of a complex of three different proteins of which one is called the Lethal Factor. The Lethal Factor of the Antrax toxin is a metallo-protease that’s able to specifically cleaves and inactives MAP kinases (Young and Collier, 2007). Another example is the botulinum neurotoxin (BoNT) from *Clostridium botulinum*. BoNT is regarded as one of nature’s most lethal toxins’s known to man with a LD50 of roughly 0.005–0.05 µg/kg (Kukreja et al., 2009). BoNT posses a metallo-protease domain which is able to block acetylcholine release at peripheral nerve ending by the cleavage the SNAP-25 protein that plays a role in the storage and depletion of acetylcholine (Dolly and Aoki, 2006).

*P. aeruginosa* also secretes several proteases that play a role in the pathogenic interaction between bacterium and host. This chapter gives an insight in the most important proteases in the weapons armoury of *Pseudomonas aeruginosa*.

**Elastase B**

**Protease classification en structure**

Elastase B is a metalloprotease belonging to the M4 thermolysin peptidase family as classified in the MEROPS database (Rawlings et al., 2008). The lasB gene codes for a preproelastase of 498 amino acids and consists of 3 different domains (figure 4.1) (Thayer et al., 1991). The first two domains represent the propeptide. The third domain harbours the catalytic thermolysin metalloprotease centre of the M4.005 subfamily (Thayer et al., 1991). The first 23 amino acids from the N-terminal code for a signal peptide that directs the protein for translocation over the inner membrane to the periplasm by the Sec system during which the signal is cleaved of by the SecB protein (Braun et al., 1998). During the translocation across the inner membrane the preproelastase is in an unfolded state. Subsequently, before the proelastase is secreted to the extra cellular environment, the protein is folded to its active and secretion competent form by the propeptide. The two domains of the propeptide act as a chaperone in which they support a correct refolding
along with other periplasmatic chaperones and folding catalysts (Braun et al., 2001). The propeptide domain is processed after correct folding by autoproteolytic cleavage and will remain non-covalently attached to the mature elastase (Kessler and Safrin, 1988). After correct folding and autoproteolytic cleavage, the propeptide-elastase complex is subsequently translocated across the outer membrane. The translocation is medicated by the Xcp machinery of the type II or the general secretory pathway (figure 4.2). A correct conformation of the protein is essential for secretion. The attached propeptide acts as an inhibitor of the mature elastase and dissociates and is degraded in the extracellular environment (Kessler et al., 1992).

The three dimensional structure of elastase has been solved and is structurally related to other solved structures of thermolysin proteins (Thayer et al., 1991). The structure of the catalytic domain confirms the presence of a single cationic zinc atom bound by three amino acids ligands His-140, His-144 and Glu-164. The two histidines 140 and 144 are part of the well known HEXXH motif for metalloproteases as refined by Jongeneel (Jongeneel et al., 1989). The glutamic acid (E) within the HEXXH motive is also present in the active site and is considered essential for catalysis (Thayer et al., 1991).

**Regulation**

Elastase is one of the prototype virulence factors of *P. aeruginosa* regulated by the quorum sensing cascade (Williams and Cámara, 2009). Quorum sensing is an important communication system involved in the pathogenicity of many bacteria. Many virulence factors of *P. aeruginosa* are regulated by quorum sensing such as proteins as exotoxin A and the non-protein virulence factors e. g. pyocyanin and rhamnolipids. The production and subsequent secretion of elastase B is regulated by quorum sensing as it is part of the las and rhl regulons (Williams and Cámara, 2009). Quorum sensing is a kind of bacterial cell-to-cell communication via small molecular chemical signals. At a certain threshold these messengers such as the group of N-acylhomoserin lactones (AHL) will bind to the transcriptional regulator LasR which subsequently activates transcription of its las regulon. Another transcriptional regulator called RhlR which is part of the las regulon also binds to certain members of the AHL signal molecules. Upon AHL binding RhlR will start transcription of its rhl regulon. The transcription is initiated by binding of the regulator to the las-rhl box
within the promoter region of the respective target genes. A las-rhl box can function as binding sites for either or both regulators (Williams and Cámara, 2009). Upstream of the lasB gene lays a LasR transcriptional binding site. Elastase B depends on both las and rhl systems as deletion mutants strains of both lasR and RhlR results in reduced and or - in the case of double mutants - in completely abolished elastolytic activity (Pearson et al., 1997). Also another system within quorum sensing network called the PQS system which is hierarchically below the las-rhl systems but all intercalate with each other and plays a role in the regulation of elastase B. The Pseudomonas quinolone signal (PQS) is a small molecular weight molecule that can bind to the transcriptional regulator PqsR (MvfR). Activation of the PQS system by addition of PQS leads to induction of lasB transcription (McKnight et al., 2000), as inactivation of the PQS signal results in decreased elastolytic activity (Sio et al., 2006).

**Role of elastase B in Host-pathogen interaction**

Elastase B was first identified as an elastolytic protease and this activity is believed to play a key role in the Cystic Fibrosis lung infection (Voynow et al., 2008). Elastase B can degrade human and bovine elastin (Hamdaoui et al., 1987; Saulnier et al., 1989). Elastin is a protein forming a biopolymer in organs and tissues of vertebrates that gives them elastic properties (Debelle and Tamburro, 1999). It is known in Cystic Fibrosis patients that the lung tissue shows decreasing levels of elastin and increasing levels of collagen resulting in pulmonary fibrosis (Voynow et al., 2008). Histologic studies have detected abnormal elastin fibers in lung alveoli of CF patients on autopsy (Bruce et al., 1985). Elastin can also be found around vascular tissue in the external elastic laminae and its disintegration is associated with vasculitis during P. aeruginosa infection (Schultz and Miller, 1974).

Elastase B is not only able to degrade elastin but also collagen - another important human biopolymer. Collagen can be found throughout a whole range of different human tissues and can be subdivided in different types. Elastase B was shown to degrade collagen type III and IV (Heck, Morihara, McRae, et al., 1986; Kessler and Safrin, 1988). Collagen type III can be found in interstitial extracellular matrixes and type IV collagen in basement membranes. Basement membranes (BM) are specialized extracellular matrices which form thin acellular layers that underlie cells and separate the cells from and connect them to their interstitial
matrix (Kruegel and Miosge, 2010). Bejarano and co-workers were able to show that elastase B was responsible for the degradation of intact basement membranes obtained from bovine lenses and lungs (Bejarano et al., 1989). The formation of BMs is a prerequisite for normal tissue development and function and regulates different biological activities during cell development. The degradation of BMs was also demonstrated in a mouse model after subcutaneous injections of purified elastase B and caused severe hemorrhage and muscle damage (Komori et al., 2001). Pathological studies showed a loss of endothelial integrity along with changes in the structure of the vascular wall. These findings were confirmed within cultured endothelial cells. The pathological effects were described to be the result to the fibrinogenolytic and fibrinolytic activity of elastase B (Komori et al., 2001).

In another study elastase B producing P. aeruginosa strains were shown to degrade proteins from human wound fluids and human skin biops ex vivo. Elastase B was also shown to inhibit fibroblast growth (Schmidtchen et al., 2003). All of these features are characteristics of chronic ulcer infections with P. aeruginosa (Schmidtchen et al., 2003).

Elastase B can also interact with proteins of the human immune defence system and degrade them. Heck and co-workers were able to show that elastase B could degrade immunoglobuline A (IgA) (Heck et al., 1990). IgA is a major serum immunoglobulin and the predominant antibody class in the external secretions that bathe mucosal surfaces such as the respiratory and gastrointestinal tract and the eye which plays a key role in the defence of invading micro-organisms (Jenny and Michael, 2006). Elastase B is also able to degrade immunoglobuline G (IgG), the most predominant and important antibody (Bainbridge and Fick, 1989). It was shown that elastase B is not only processed but that the degradation products of IgG obtain from CF patients inhibit bacterial uptake by human neutrophils and therefore prevent opsonophagocytosis (Bainbridge and Fick, 1989). So the action of elastase B is ambiguous, first it inactivates IgG and secondly the inhibition of bacterial uptake by phagocytosis of the degradation products. Phagocytosis is probably inhibited because of the binding of the degradation productions to the IgG receptors on the human neutrophils preventing uptake of bacterial invaders bound by IgG as an opsonin (Bainbridge and Fick, 1989).
Proteases and their role in virulence of *Pseudomonas aeruginosa*

Figure 4.1: Schematic gene structure of the major virulence proteases of *P. aeruginosa*, elastase B (LasB), elastase A (LasA), protease IV (PIV), alkaline protease (AprA). Red bars represent signal peptide sequence (SP), green propeptide domain and in blue the peptidase domain (numbers are MEROPS peptidase classification) (Hoge et. al, 2011).
As part of the innate immune system the complement system eliminates intruding pathogens (Pangburn et al., 2008). Elastase B is able to inactivate key components of the complement system such as fluid-phase and cell-bound C1 and C3 and fluid-phase C5, C8 and C9 (Schultz and Miller, 1974).

Another part of the innate immune system is inactivated by Elastase B in its ability to degrade Surfactant Protein A and D (SP-A and D) (Mariencheck et al., 2003). Surfactant Proteins, members of the collectin family, are synthesized by alveolar type II epithelial cells and consists of an N-terminal collagen region and a C-terminal lectin domain. Complexes of the SPs or collectins are pattern recognition molecules that bind oligo saccharides presented on the surface of many bacteria and by doing so indentify them as alien intruders. SP-D binds among others *P. aeruginosa* cells. Most often this SP attachment results in the aggregation of the organisms although aggregation of *P. aeruginosa* seems to be strain depended (McCormick et al., 2007). SP-D increases the up-take of *P. aeruginosa* by macrophages as shown in vitro. There are several reports that SP-A and SP-D levels are decreased in bronchoalveolar lavage (BAL) within the CF lung and the SP-A and SP-D degradation fragments were detectable (Alcorn and Wright, 2004). McCormick and co-workers showed also a role for SP-D in *P. aeruginosa* keratitis in vivo. Deficient SP-D mice were more susceptible to cornea infection after inoculation with *P. aeruginosa* compared to wild-type animals, only the wild-type mice recovered completely of the infection (McCormick et al., 2007). Besides the respiratory tract, SP-D can also be found in tear fluid and cornea. Elastase B was suggested to be responsible for the SP-D degradation in the eye, as was Protease IV. *P. aeruginosa* is a significant cause of corneal infections. Elastase B was shown to cause damage to the stromal proteoglycan extracellular matrix after topical application or injection of wounded cornea with purified protease in different studies (Kessler et al., 1977; Kreger and Gray, 1978; Twining et al., 1993). Elastase B deficient strains were shown to be less virulent during keratitis after injection to guinea pig cornea. Co-infection of the cornea of elastase B producing strains with elastase B antibodies or the protease inhibitor ovomacroglobulin showed a significant infection reduction (Ijiri et al., 1993). Kessler and co-workers were also able to show that the elastase B inhibitor phosphoramidon could protect the corneas of rabbits for elastase B damage (Kessler and Spierer, 1984).

The elastase B corneal virulence was also shown in an assay by expressing recombinant elastase B in the non-pathogen *Pseudomonas putida* and subsequent infection of rabbit
cornea. Corneal damage was significantly increased in the *P. putida* expression the elastase B in comparison to the strain without the elastase B (Thibodeaux et al., 2007).

Elastase B is also able to inactivate the Proteinase-activated receptor-2 (PAR2). PAR2 belongs to a family of G-coupled transmembranic receptors which can be activated after cleavage at an activation site within the N-terminus of their own exodomain by a variety of proteases (Dulon et al., 2005). After cleavage, this small peptide functions as a tethered ligand which binds intramolecularly to the receptor and activates it. PAR2 is functionally expressed in different tissues among them the respiratory epithelium. Activation of PAR2 leads to a variety of responses such as triggering the secretion of prostanoids, cytokines and metalloproteases. *In vivo* activation leads to modulation of bronchomotor activity as shown in guinea pigs, neutrophil extravasation and oedema in rats and eosinophil infiltration in mice (Dulon et al., 2005). It was postulated that secreted bacterial proteases are the activators of the PAR2 receptor as thereby initiating a defence response. *In vitro* experiments with cell cultures show that elastase B is able to disarm the PAR2 receptor by cleaving within the exodomain without activating the receptor and therefore prevent a response shown by a lower secretion of interleukin-8 (IL-8) and prostaglandin E$_2$ (PGE$_2$) (Dulon et al., 2005).

Many CF patients suffer from chronical *P. aeruginosa* lung infections in which adherence of the bacteria to the lung tissue is essential. Elastase B was able to increase adherence of *P.aerugionsa* in an assay with cultured human lung fibroblasts. This increased adherence was mediated by degradation of fibronectin (FN) on the cell surface and decreased FN receptors or so called Integrins. FN can be a ligand for a dozen members of the integrin receptor family. Integrins are structurally and functionally related cell-surface receptors that link the extracellular matrix with the intracellular cytoskeleton of cells (Pankov and Yamada, 2002).

Elastase B can also cleave the urokinase-type plasminogen activation receptor (uPAR) purified after recombinant expression or expressed in human monocytic and bronchial epithelial cell lines (Leduc et al., 2007). The uPAR (also designated CD87 or Mo3 antigen) is a highly glycosylated glycosylphosphatidylinositol (GPI)-anchored cell membrane protein which is composed of three domains D1, D2 and D3. Its expression is increase upon the exposure of cells to a wide range of inflammatory mediators. uPAR has a high affinity for different types of proteins that are involved in cell adherence and migration. uPAR can e. g. bind the serine protease uPA (urikinase-type Plasmingen Activator) which on binding
converts plasminogen into plasmin. uPAR-deficient mice are impaired in recruitment and activation of leukocytes at sites of infection, resulting in impaired bacterial clearance and increased host mortality. Besides that uPAR-deficient animal models may indicated its role in tissue repair and healing after an inflammatory event (Leduc et al., 2007). Elastase B can inactivate the binding of uPAR to substrates by cleavage at several different parts of the protein.

Research done on guinea pigs showed an increase of lung epithelial permeability after treatment with elastase B aerosol respiration. This epithelial permeability was the result of the disruption of the tight junctions between epithelial cells (Azghani et al., 2000). The same researcher showed that elastase B could enhance IL-8 production in rabbit alveolar epithelial cells by activation of the mitogen-activated protein kinase (MAPK) pathway via extracellular signal-regulated (ERK1/2) proteins (Azghani et al., 2002). This activation was abolished in the presence of the ERK activation inhibitor U0126.

Human α1-proteinase inhibitor (α1-PI) is responsible for the thigh control of elastase activity secreted from neutrophils by inhibiting it. Down regulation of α1-PI may cause excessive tissue degradation. Elastase B from P. aeruginosa is able to inactivate α1-PI by cleavage (Morihara et al., 1984).

In rats elastase B can induce neutrophil accumulation after injection in the air-pouch cavity of the respiratory tract. After injection of elastase B the volume of exudates and neutrophil amounts were increased significantly and showed a peak 8 hours after infection. IL-8 level were highest 4 hours after infection that which production were also enhanced by elastase B (Kon et al., 1999).
Proteases and their role in virulence of *Pseudomonas aeruginosa*

Figure 4.2: Schematic secretion of the major virulence proteases of *P. aeruginosa*, elastase B (LasB), elastase A (LasA), protease IV (PIV), alkaline protease (AprA). Abbreviations are cytoplasmic membrane (CP), inner membrane (IM), periplasm (PP), outer membrane (OM), extra cellular (EC), ferric uptake regulator (Fur), alternative sigma factor (PvdS), signal peptide (SP), Secretory pathway (Sec), Xcp machinery (Xcp), alkaline secretion proteins (AprD, AprE and AprF), propeptide domains (p) and catalytic domains or mature proteases (c). Scissors represent (auto)proteolytic activity and arrows indicate the direction of secretion. Hoge et al., 2011.
Chapter 4

Elastase A

Protease classification and structure

Elastase A (LasA), also known as staphylolysin, is one of the most abundant of at least four secreted endopeptidases of *P. aeruginosa*. It was originally identified by a mutation (*lasA1*) in strain PAO1 that results in reduced elastinolytic activity (Ohman et al., 1980). Elastase A is a zinc metallopeptidase (Kessler et al., 1997) and belongs to the subgroup A of M23 family of staphylolysic or β-lytic zinc metallo-endopeptidases (Rawlings and Barrett, 1995; Rawlings et al., 2010b). These enzymes are able to cleave several glycine-containing proteins, one of the substrates is elastin and, in addition, they are able to cause lysis of *S. aureus* cells (Kessler et al., 1993). The mechanism of action of M23A endopeptidases and the three-dimensional structures are apart from LasA not known. LasA is the first member in this family which three-dimensional structure was solved (Spencer et al., 2010). As a member of the M23 metalloproteases, LasA possesses a characteristic His-x-His motif (Hooper, 1994). The presence of these two histidine residues in the active site are a typical feature of M23 metalloprotease. In the case of LasA the histidine positions are His81 and His120 that are highly conserved but both histidines are not required to coordinate the Zn$^{2+}$. Mutagenesis studies on LasA and other M23 family members already showed that these two histidines are necessary for activity (Gustin et al., 1996). The molecular interactions made by these residues during catalysis have recently been analyzed (Spencer et al., 2010).

The *lasA* gene encodes a 40 kDa protein (Schad and Iglewski, 1988) which represents the elastase A preproprotein. The mature LasA that can be found in the extra cellular environment has a molecular weight of 20 kDa (Gustin et al., 1996; Schad and Iglewski, 1988). After its synthesis LasA is secreted together with its propeptide via the Xcp (type II) machinery (Braun et al., 1998; Kessler et al., 1998) and is subsequently activated when it has reached the extracellular space (Kessler et al., 1998). The determination of the 31 amino acids long authentic signal peptide was enabled by *lasA* expression in *E. coli* cells and subsequent N-terminal sequence analysis (Gustin et al., 1996).

A definite prediction of the three domain sizes was possible in comparison with the sequence data. The propeptide possesses 205 amino acids and is thus 23 amino acids longer than the mature LasA protease with 182 amino acids. The signal peptide is cleaved as the protein is translocated across the cytoplasmatic membrane and, as it is the case also for
elastase B, the propeptide remains covalently bound until the protein is secreted from the cell (Kessler et al., 1998). The fact that the propeptide is as long as the mature protease has been documented for other proteases, e.g. the β-lytic protease of Achromobacter licus (Li et al., 1990). Several bacterial proteases require a covalently bound N-terminal propeptide to fold them into an active conformation. Furthermore this propeptide region inhibits protease activity and it influences stability and even secretion of the protein. Such propeptides are termed intramolecular chaperone (IMC) (Chen and Inouye, 2008; Shinde and Inouye, 2000).

The detailed function of the LasA propeptide has to be determined but it is reasonable to assume that the propeptide may also function as an IMC that is responsible for folding of LasA to obtain the mature and enzymatically active protease. A current mechanism for cleavage of the propeptide that leads to the release of the active protease is autoproteolysis. The propeptide of P. aeruginosa LasB is a paradigm for a classic IMC (Braun et al., 1996). It is critical for secretion and activation of the mature protease as mentioned before (McIver et al., 1995; McIver et al., 1993). The propeptide is cleaved by LasB itself autoproteolytically (McIver et al., 1991; McIver et al., 1993). In contrast to LasB the cleavage of the LasA propeptide is not autocatalytic (Gustin et al., 1996). Instead, the LasA propeptide is processed by different endopeptidases secreted by P. aeruginosa named LasB, LysC and protease IV (Engel,Hill,Caballero, et al., 1998; Wilderman et al., 2001). The propeptide sequence of LasA in principle shows little identity to those of other β-lytic proteases. Therefore it is difficult to predict functional domains within the propeptide, e.g. the secretion signals directing proLasA into the Xcp (type II) machinery could be within the propeptide sequence but it has still to be identified.

The crystal structure of LasA in complex with tartrate was recently solved (Spencer et al., 2010). LasA is the first member of M23A proteases that has been structurally characterized. The uncovered structure of LasA represents a three-layered sandwich of antiparallel β-sheets with a central sheet of eight strands that is flanked by two shorter sheets composed of three and four strands each. The essential zinc ion is arranged at the bottom of a deep groove coordinated by conserved His and Asp protein ligands. LasA shares high homology with the mature domains of the β-lytic proteases of Achromobacter lyticus and Lysobacter enzymogenese (both approximately 40 %). This group of β-lytic endopeptidases can cause cell lysis of other bacteria by cleaving specific peptide bonds within the cell wall.
peptidoglycan network (Kessler, 1995; Kessler et al., 1993) and includes enzymes like the β-
lytic protease of *Achromobacter lyticus* as an example (Li et al., 1990).

**Role of elastase A in Host-pathogen interaction**

LasA has both low elastinolytic and high staphylolytic activities (see below) (Kessler et al., 1993). LasA cleaves a wider range of glycine-containing proteins, including tropoelastin-derived pentapeptides (Kessler et al., 1997), glycine-rich synthetic peptides and specific sequences present in elastin (Kessler et al., 1997). Such sequences are rare in elastin resulting in the limited elastinolytic power of LasA protease (Vessillier et al., 2001). LasA shows activity towards branched or aromatic amino acids at the P1’ position. These substitution are less favored at P2’ position. At P1 position the amino acid glycine was preferred. According to the nomenclature of Schechter and Berger (Schechter and Berger, 1967, 1968) P1 illustrates the first amino acid residue in the N-terminal direction of the cleaved peptide bond. The first amino acid residue on the carboxyl side of the cleavage site is labeled P1’. Peptide sequences like LGGGA which are cleaved by LasA are present in several human proteins. These sequences are present e.g. in the ras p21 GTPase activating protein, in protocadherin, in some immunoglobulin variable regions, in the adenylate cyclase activating protein and in several further proteins (Vessillier et al., 2001) which may imply that LasA may have far more target proteins in addition to elastin and may thus play a significant role in *P. aeruginosa* virulence establishment. Elastin is an important component of connective tissues, blood vessels and lung tissue. In comparison to LasB the elastinolytic power of LasA is rather limited (Kessler et al., 1997; Peters and Galloway, 1990; Wolz et al., 1991). Besides the own intrinsic elastinolytic activity, LasA increases significantly the elastinolytic activity of other proteases, including that of LasB (Kessler et al., 1997; Peters and Galloway, 1990; Toder et al., 1991). Therefore LasA enhances the virulence activity of LasB in the establishment of a *P. aeruginosa* infection. Moreover, LasA enhances also the activity of several other host elastinolytic proteases, including human leukocyte elastase, human neutrophil elastase (Peters et al., 1992) and other proteases (Kessler et al., 1997; Peters and Galloway, 1990). This effect on host proteins is possibly of importance to lung pathology associated with *Pseudomonas* infections (Peters and Galloway, 1990). The concerted action of several enzymes in elastin degradation and the relationship
Proteases and their role in virulence of *Pseudomonas aeruginosa*

of the two *Pseudomonas* elastases LasA and LasB appear to be involved in generating the invasive phenotype of some *P. aeruginosa* strains (Cowell et al., 2003; Kessler et al., 1997; Peters and Galloway, 1990; Wolz et al., 1991).

Elastase activity of *P. aeruginosa* has effects on tight junctions of epithelial cells (Fleiszig et al., 1997). The loss of either LasB or LasA decreases *in vitro* invasion of epithelial cells about 70% and the loss of both proteases leads to a further significant decrease of the ability to invade host cells (Cowell et al., 2003). Inactivation of the *aprA* gene encoding for the alkaline protease in addition to *lasA* and *lasB* mutation, however, did not further decrease invasion of epithelial cells. These results indicated that both LasA and LasB might play critical roles in the regulation of tissue invasion (Cowell et al., 2003). One opportunity by which LasA and LasB may induce invasion could be the degradation of inhibitors of invasion or of degradation of proteins involved in biosynthesis, processing and delivery of invasion inhibitors. The secreted effector proteins ExoS and ExoT are able to inhibit the invasion of epithelial cells by cytotoxic strains of *P. aeruginosa*. There are indications that LasA and LasB directly or indirectly decrease the levels of the toxins ExoS and ExoT that leads to a reduction of invasion inhibition (Cowell et al., 2003).

In addition to the protease activity of LasA against substrates like elastin the protease possesses staphylolytic activity. This staphylolytic activity causes rapid lysis of *S. aureus* cells by cleaving the pentaglycine bridges of their cell wall peptidoglycan. The overall staphylolytic activity of *P. aeruginosa* is mainly LasA dependent. LasA-induced lysis of *Staphylococci* is stimulated and enhanced by other proteases like LasB or the alkaline protease (Gustin et al., 1996). Physiologically the staphylolytic activity of *P. aeruginosa* may represent a defence strategy against a competing organism and may give *P. aeruginosa* an important advantage to outcompete *S. aureus* during colonization of the cystic fibrosis lung (Bernhardt and de Boer, 2004; Smith et al., 2000).

One novel approach to utilize the staphylolytic activity of LasA is to use the protease per se as a novel antibacterial tool for the treatment of methicillin-resistant *S. aureus* endophthalmitis (Barequet et al., 2009). It was shown that LasA is effective in the treatment of MRSA-induced endophthalmitis in an experimental model in rats. Furthermore the protease causes no further damaging effects to ocular tissues. However, to further determine the therapeutic potential of LasA, continuative studies concerning to LasA activity
towards additional S. aureus strains and possible clinical and functional effects have to be performed.

Beside the already mentioned interactions of LasA with host proteins like elastin the influence of LasA on the so called shedding process is of particular importance to enhance pathogenesis of P. aeruginosa. The shedding process constitutes the cleavage of cell surface proteins by proteases and the release of ectodomains from the surface as soluble effectors (Arribas et al., 1996; Hooper et al., 1997). Shedding is an elemental biological mechanism of protein secretion. Surface molecules like growth factors and growth factor receptors, adhesion molecules and cytokines are shed as soluble ectodomains. Shed ectodomains are involved in several pathophysiological events like tissue repair, host defense, septic shock, Alzheimer’s disease and wound healing processes (Hooper et al., 1997; Isberg and Leong, 1990; Kato et al., 1995; Relman et al., 1990). P. aeruginosa seems to use the host cell’s shedding mechanism to increase its virulence.

One family of cell surface transmembrane glycoproteins is termed syndecans which has four members (Bernfield and Sanderson, 1990; Salmivirta and Jalkanen, 1995). Syndecans possess a heparin sulfate chain which enables binding and modulation of the activity of a several soluble and insoluble ligands. The extracellular part of syndecan can be shed and after the release as soluble ectodomains they can function as soluble effectors. The constitutive shedding process of syndecan is a normal host-regulated mechanism. Besides there are hints that syndecan shedding is induced by tissue injury. This host-regulated response leads to enhanced shedding and the released ectodomains function as regulators of inflammation (Subramanian et al., 1997). During infection secreted virulence factors of bacteria enhance host ectodomain shedding that leads to epithelial barrier disruption, tissue penetration and endothelial damage (Park et al., 2001; Park et al., 2000). One of these secreted virulence factors is LasA of P. aeruginosa. LasA enhances shedding of syndecan-1 in vitro (Park et al., 2000) and also in vivo (Park et al., 2001). The released soluble syndecan-1 ectodomains subsequently enhance bacterial virulence through their heparan sulfate chains in newborn mice (Park et al., 2001; Park et al., 2000). Keeping in mind that P. aeruginosa is a dominant pathogen in cystic fibrosis and burn wounds it is not surprising that syndecan-1 is the major syndecan of the lung epithelia and epidermal keratinocytes cells. The exact reason for enhancement of P. aeruginosa virulence because of the heperan sulfate chains remains to be determined. It is clear so far that the ectodomains do not interact directly with
P. aeruginosa (Park et al., 2001). It was suggested that shed ectodomains may promote bacterial pathogenicity by interfering with host defense system (Park et al., 2001). There are several hints that soluble, distinct host defense factors like neutrophil elastase and cathepsin G (Kainulainen et al., 1998) are inhibited by shed syndecan-1 in turn facilitating the attack of P. aeruginosa.

**Protease IV**

**Protease classification and structure**

Protease IV was first indentified and characterized as a 26 kDa serine protease of the culture supernatant of P. aeruginosa (Elliott and Cohen, 1986). Protease IV is also known as lysyl endopeptidase or iron-regulated protein PrpL and belongs to the chymotrypsin family S1 according to the MEROPS database (Rawlings et al., 2010a). The catalytic domain contains three active residues His-72, Asp-122 and Ser-198 that form a triad in the catalytic cavity predicted based on homology with other proteases of this family and proven by mutagenesis studies (Traidej, Marquart, et al., 2003). The serine (Ser-197) adjacent to Ser-198 was also shown to be essential for catalytic activity. The piv gene, however, encodes a protein of 463 amino acids with a calculated size of 48.2 kDa. The piv gene consist of three domains; a short N-terminal secretion signal followed by a propeptide and a C-terminal catalytic domain. The protease is synthesized intracellular as a pre-proenzyme of 48 kDa. Bioinformatic analysis predicts a 24 amino acid long signal peptide. Expression studies with the whole open reading frame of Protease IV in P. putida confirms the presence of a proenzyme of about 45 kDa (Traidej, Caballero, et al., 2003). The mature protease could not be detected in the supernatant after expression in P. putida although proteolytic activity was detected in cell lysates. In comparison to other proteases such as elastase B it is likely that the pre-proenzyme is translocated across the inner membrane to the periplasm after which the signal peptide is cleaved of. In the periplasm the propeptide is probably removed by autoproteolysis thereby liberating the mature protein. Evidence for autoproteolysis was shown by processing of the inactive mutated proenzyme His-72-Ala after addition of purified Protease IV (Traidej, Caballero, et al., 2003). It was suggested that autoprocessing involving the cleavage of the 45 kDa proenzyme takes place at the lysine residue at the junction between the propeptide and the mature protease domains (figure 4.1). The function of the propeptide is
unknown but probably it acts as a intermolecular chaperone in analogy to elastase B and assists in folding of the mature protease, secretion of the enzyme and it may have an inhibitory function. Traidej and coworkers hypothesized that Protease IV could be secreted by the type II secretion apparatus for translocation across the outer membrane (Traidej, Caballero, et al., 2003).

## Regulation

The expression of Protease IV has been shown to be regulated by the alternative sigma factor PvdS because it was absent in culture supernatants of a *P. aeruginosa* PvdS deletion mutant (Wilderman et al., 2001). PvdS belongs to the extracytoplasmic factor class of regulatory proteins and regulates virulence genes such as Exotoxin A (Wilderman et al., 2001). The PvdS itself is regulated by the ferric uptake regulator (Fur) which contributes to the expression of many virulence factors. The human host iron-binding proteins lactoferrin and transferrin are normal constituents of airway secretion which are important in host defenses by limiting the availability of iron as essential element. Iron concentration can be increased by degradation of transferrin and lactoferrin and their subsequent iron release.

Britigan and coworkers showed that cleavage products of transferrin and lactoferrin could be detected in the bronchoalveolar lavage (BAL) of the lungs of CF patients that were not present in normal individuals. These products were only detected in CF patient in whom *P. aeruginosa* was determined (Britigan et al., 1993). Wilderman and coworkers showed that the expression of Protease IV is enhanced on iron limitation and that Protease IV is able to cleave lactoferrin and transferring (Wilderman et al., 2001).

Protease IV production appears also to depend on other factors. *P. aeruginosa* produces two lectins LecA and LecB which have been shown to play a role in virulence (Bartels, 2010). Sonawane and coworkers showed that the lack of LecB results in a decrease Protease IV activity (Sonawane et al., 2006). This suggests a coupling of protease IV expression to the production of other virulence factors, although the regulatory mechanism of this coordinated expression is not known at present.
Enzymology

Protease IV cleaves proteins predominantly at the carboxy-terminal side of lysine residues but resistant to the amino acids combination Lys-Pro (Elliott and Cohen, 1986). Protease IV can be inhibited by N²-p-tosyl-L-Lysine chloromethyl ketone (TLCK) which alkylates histidine residues. This is in agreement with the function of His-72 which is part of the catalytic triad residues and potential target for TLCK. Protease IV was only partially inhibited by the serine inhibitors diisopropyl fluorophosphates and phenylmethylsulfonyl fluoride (Elliott and Cohen, 1986). The reducing agents dithiothreitol and 2-mercapto could also inhibit protease activity (Engel,Hill,Moreau, et al., 1998).

Role of protease IV in host-pathogen interaction

Protease IV is able to cleave bovine fibrinogen in vitro (Elliott and Cohen, 1986) which is a large biopolymer and part of the blood clotting system (Walsh and Ahmad, 2002). Fibrinogen is conferred to a fibrin cloth after vascular damage. Dysfunction of fibrinogen will lead to hemorrhage which is a characteristic of P. aeruginosa infection (Elliott and Cohen, 1986). Engel and coworkers showed that Protease IV could degrade a whole range of biological important host proteins such as fibrinogen and plasminogen system and immunoglobulin G (IgG) and the complement components 3 and C1q all belonging to the immune defence system (Engel,Hill,Moreau, et al., 1998).

Da Silva and coworkers showed that P. aeruginosa is able to bind human plasminogen (Plg) to his extra cellular surface (da Silva, 2004). Plasminogen plays an important role in the delicate equilibrium of blood clotting. Plasminogen can be activated by other proteases such as the urokinase-type (uPA) proteolytic activator by conversion into the active plasmin. Plasmin is a serine protease that degrades a fibrin cloth. It is thought that plasminogen can also have other physiological functions that may be misused by pathogenic bacteria. Several human cells bind Plg that assists and directs migration through out the body (da Silva et al., 2004). Pathogenic bacteria, among them P. aeruginosa can bind Plg which helps them to invade the whole host and the attached protease can degrade extracellular matrix (ECM) proteins or helps in escaping from fibrin networks that serve as a focus of infection (da Silva et al., 2004). In order to be effective the bound Plg has to be converted into the active plasmin (Pm). Beaufort and coworkers showed that Plg could not be activated by elastase B.
or protease IV directly, but Protease IV is able to cleave pro-uPA into active form uPA. uPA on his turn will convert Plg in Pm and therefore activates it (da Silva et al., 2004).

Like elastase B protease IV is also able to degrade several different surfactant proteins (SP) such as SP-A, SP-D and SP-B. Malloy and coworkers showed that Protease IV is able to degrade SP-A, SP-D and SP-B in a time- and dose depended fashion in cell-free bronchoalveolar lavage fluid (BAL). This degradation could be inhibited by TLCK (Malloy et al., 2005). In the course of the original observation that \textit{P. aeruginosa} was able to cleave SP-A and SP-D it was suggested that two different proteases were responsible (Mariencheck et al., 2003). During this study only one protease, elastase B, was identified as a responsible protease. Later on protease IV could be identified in a follow-up study as the second protease (Malloy et al., 2005). The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages. Furthermore it was shown that the surface tension lowering effect of large surfactant aggregates (LA) was abolished by Protease IV (Malloy et al., 2005). This effect on LA could be inhibited by TLCK. Protease IV thus alters both functions of the surfactant proteins; surface tension reduction and innate immune system. In addition to elastase B, Protease IV can also degrade SP-B in BAL. As mentioned before SP-D was shown to play a role in \textit{P. aeruginosa} keratitis (McCormick et al., 2007).

Most research on the virulence of protease IV has been done in elucidating its role in keratitis of the cornea (Caballero et al., 2004; Matsumoto, 2004). Protease IV was identified in attenuated and deficient \textit{P. aeruginosa} strains to contribute to the virulence of corneal infection in rabbits and mice (Engel et al., 1997; O’Callaghan et al., 1996). These results could be confirmed by restoring the virulence in a keratitis rabbit infection model by co-applying purified Protase IV together with a Protease IV deficient \textit{P. aeruginosa} strain (Engel,Hill,Moreau, et al., 1998). Thibodeaux and coworkers investigated the protective value of immunization against Protease IV in preventing keratitis in rabbits (Thibodeaux et al., 2005). For this rabbits were immunized with purified Protease IV and urea-soluble recombinant Protease IV. Antibody titers were raised against recombinant Protease IV and purified Protease IV. Nevertheless the antibodies could not neutralize enzyme activity \textit{in vitro} neither protect infected rabbits in developing keratitis. Marquart and coworkers reported that additional calcium amount in media could enhance Protease IV activity in these cultures. Calcium concentrations measured in corneas of rabbits infected with \textit{P.
aeruginosa were increased indicating that this elevation can enhance virulence of Protease IV (Marquart et al., 2005).

Protease IV has also been shown to be involved in the virulence of a P. aeruginosa infection in Galleria mellonella. Larvae of the Great wax moth G. mellonella have been used as a non-mammalian hosts to study the virulence of P. aeruginosa for decades (Jander et al., 2000) P. aeruginosa is highly virulent for G. mellonella larvae as LD50 values ranges from 2 to 20 bacteria. Protease IV has been shown to degrade in vitro the Apoliphorin III (apoLp-III) a major protein found in the haemolymph that plays an important role in the insect innate immune response (Andrejko, 2004). Follow-up studies showed that apoLp-III degradation in vivo after infection of G. mellonella is caused by Protease IV activity (Andrejko et al., 2005).

In response to alien intruders hosts have developed different mechanisms to evade attacks by proteases. Bellemare and coworkers reported that the human pre-elafin protein inhibits Protease IV activity (Bellemare et al., 2008). Pre-elafin is a protein constraining two domains: an N-terminal domain termed cementon and a C-terminal domain with is homologues to the whey acidic protein (WAP). In the lung pre-elafin is expressed by alveolar mactrophages and epithelial type II cells and has inhibitory effects on neutrophil elastase. Pre-elafin could inhibit Protease IV activity as efficiently as TLCK (Bellemare et al., 2008).

Alkaline protease

Protease classification en structure

The alkaline protease (AprA), also known as aeruginolysin, is one of the secreted zinc-dependent metallo-endopeptidase of P. aeruginosa. AprA belongs to the subfamily B of the M10 peptidase family and is a member of the so called metzincin superfamily (Rawlings et al., 2010b). Proteases of the metzincin superfamily possess the characteristic zinc binding motif HEXXHXXGXXH and the active sites of these proteases contain a conserved methionine-containing 1, 4-tight β-turn (Bode et al., 1993). The zinc ligands are His185, His189 and His195 and the catalytic residues Glu186 and Tyr225 are localized in the active site cleft within the N-terminal domain of the protein (Baumann et al., 1993). AprA was originally characterized by Morihara, has a molecular weight of 50 kDa (Morihara, 1963;
Morihara et al., 1965) and its activity optimum is in the alkaline pH range of 8-10 (Morihara et al., 1973). Upon secretion the inactive precursor is activated by processing of nine N-terminal residues resulting in the mature protease of 470 amino acid residues (Okuda et al., 1990). The C-terminal domain binds several calcium ions which are essential for stabilization of the protein (Morihara and Tsuzuki, 1964) in a mainly structural β-role domain. The structure of the β-role domain depends on the glycine-rich sequence motif GGXGXDX(L/F/I)X (Lilie et al., 2000). The sequence motif is tandemly repeated and finally forms a calcium binding structure with 21 consecutive β-strands arranged in a right-handed spiral in which eight Ca$^{2+}$ ions are coordinated (Baumann et al., 1993; Lilie et al., 2000). The AprA crystal structure was determined using a complex of the enzyme with a mixture of tetrapeptide products (Baumann et al., 1993). The crystal structure of the unliganded alkaline protease has been determined afterwards (Miyatake et al., 1995).

The apr locus of P. aeruginosa contains five open reading frames and encodes for proteins that are involved in either the synthesis or secretion of the alkaline protease (Guzzo et al., 1990) among them the gene aprA, encoding for the alkaline protease itself and the gene aprl, which encodes the protease inhibitor APRin. The three proteins AprD, AprE and AprF are membrane proteins that are necessary for AprA secretion. The genes encoding these membrane proteins are clustered adjacent to the 5’ end of the alkaline protease structural gene aprA (Duong et al., 1992; Guzzo et al., 1991). The AprD, AprE and AprF proteins show a high homology to proteins necessary for secretion of proteases from Erwinia chrysanthemi and to the α-haemolysin of Escherichia coli (Duong et al., 1992) and form the type I secretion machinery for translocation of alkaline protease (Guzzo et al., 1991). AprF is localized within the outer membrane, AprE functions as a membrane fusion protein and AprD represents the ATP-binding cassette. Like all proteins secreted by this type of mechanism, AprA possesses a very characteristic C-terminal secretion signal located within the last 50 amino acids residues (Duong et al., 1996). The exact properties of the signal sequence haven’t been determined yet.

As already mentioned, the apr locus of P. aeruginosa encodes also for an alkaline proteinase inhibitor (APRin). This 11.5 kDa inhibitor protein shows high affinity and high specificity towards AprA (Arumugam et al., 2008). APRin consists of 131 amino acid residues with a signal peptide of 25 residues. The signal peptide is cleaved off during translocation across
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the inner membrane. The crystal structure of APRin was solved (Arumugam et al., 2008; Hege et al., 2001) and by the structure analysis of a protease-APRin complex it was possible to show that the coordinative interaction of APRin and alkaline protease occurs between the N-terminus of the inhibitor and the catalytic Zn$^{2+}$ ion of the alkaline protease (Hege et al., 2001). Especially the five N-terminal amino acid residues of the inhibitor bind the extended substrate binding site of alkaline protease (Feltzer et al., 2000; Hege et al., 2001). In kinetic assay it was demonstrated that these residues are necessary for strong reversible binding of APRin to the alkaline protease (Feltzer et al., 2000). One of these five amino acid residues, the terminal serine, coordinates to the catalytic Zn$^{2+}$ ion of the alkaline protease (Hege et al., 2001). Elastase B is able to cleave the APRin which leads to removal of 1–5 amino acid residues from the N-terminal resulting in the inactivation of the inhibitor (Feltzer et al., 2000). The physiologically function of APRin is not clear in detail but it is suggested that APRin is essential during alkaline protease secretion. Presumably APRin is co-secreted from the bacterium to inhibit proteolysis of host proteins by the alkaline protease (Arumugam et al., 2008; Letoffe et al., 1989). Beside this natural inhibitor it was shown that EDTA and o-phenanthroline can effectively inhibit alkaline protease activity (Morihara, 1963).

**Role of alkaline protease in host-pathogen interaction**

It was shown that aeruginolysin cleaves a large number of physiological substrates *in vitro* (Matsumoto, 2004). One of these substrates is laminin which is an important and biologically active part of the basal lamina (Heck, Morihara and Abrahamson, 1986). Soluble laminin is rapidly cleaved by purified *P. aeruginosa* alkaline protease. Laminins are big trimeric proteins that contain a α-chain, a β-chain and a γ-chain. Aeruginolysin rapidly cleaves the α-chain but slowly cleaves the β-chain (Heck, Morihara and Abrahamson, 1986) while degrading of both α-chain and β-chain by LasB occurs rapidly. Because of laminin degradation, alkaline protease could probably have a direct function in invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa*.

Alkaline protease and elastase B of *P. aeruginosa* inactivate human γ-interferon and human tumor necrosis factor-α (Horvat and Parmely, 1988; Parmely et al., 1990). Both cytokines are critical for effective host immune responses. γ-interferon is the key factor for innate and adaptive immunity against viral and bacterial infections and for control of tumor formation.
A lack of γ-interferon results in autoinflammatory and autoimmune diseases (Schoenborn and Wilson, 2007; Schroder et al., 2004). Tumor necrosis factor-α is a important factor for the regulation of immune cells. It is involved in systemic inflammation and is able to stimulate the acute phase reaction by induction of apoptotic cell death. Apoptosis is induced to initiate inflammation and to inhibit tumorigenesis and viral replication (Gaur and Aggarwal, 2003; O'Shea et al., 2002).

Both alkaline protease and Elastase B are able to inhibit the function of neutrophils, especially interfering with their chemotaxis (Kharazmi et al., 1984) which gives the bacterium an advantage in escaping from phagocytes of the host defense system. Another component of the host immune system that is affected by proteases of P. aeruginosa are leucocytes. It was shown that alkaline protease and LasB reduce their phagocytic activity against P. aeruginosa (Kharazmi et al., 1986). It was assumed that this effect of P. aeruginosa is based on cleavage of the cell receptors on the cell surface which are necessary for phagocytosis (Kharazmi et al., 1986). A further factor which is influenced by both proteases in vitro is the Natural Killer (NK) cell function. Alkaline protease and LasB are able to inhibit the effector/target cell conjugate formation. The inhibition of NK cell binding to the target cell is probably caused by the protease activity that enables the cleavage of surface receptors associated in the binding (Pedersen and Kharazmi, 1987). Beside the already mentioned substrates it was demonstrated that aeruginolysin can also cleave a variety of physiological substrates in vitro. These substrates are fibrin, fibrinogen and different complement factors, especially C3 (Shibuya et al., 1991). Moreover aeruginolysin is able to inactivate different human protease inhibitors such as serpins, the C1-inhibitor and α-1-antichymotrypsin, IgG from rat (Doring et al., 1984), the human RANTES, epithelial neutrophil-activating protein-78 (ENA-78) and monocyte chemotactic protein 1 (MCP-1) (Leidal et al., 2003). Furthermore it was demonstrated that elastase and alkaline protease were capable to interfere with human lymphocyte function, assumedly based on degradation of IL-2 (Theander et al., 1988).

Interestingly a second modified form of aeruginolysin with the two additional residues leucine and lysine at the N-terminus (Leu-Lys-aeruginolysin) was characterized (Matheson et al., 2006). This modified form probably derives by differential processing of the propeptide. This alternative cleavage of the propeptide may occur in vivo during the infection processes. Concerning to IL-8-77, activity tests of the Leu-Lys-aeruginolysin confirm that there is no difference in the protease activity compared to normal AprA. There was no significant
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The proteolytic activity of Leu-Lys-aeruginolysin depends on calcium concentrations. This form of aeruginolysin was less effective for IL-8-72 cleavage in the presence of calcium. As already mentioned, aeruginolysin possesses a large C-terminal calcium-stabilized structural domain (Baumann et al., 1993; Hege et al., 2001; Miyatake et al., 1995). The reason for enhanced activity of Leu-Lys-aeruginolysin and the effect of calcium are not obvious from the atomic structure of the protein. The N-terminus is arranged next to the calcium-binding structure in the three-dimensional structure (Baumann et al., 1993). It was assumed that the elongated N-terminus by the two amino acid residues leucine and lysine affects in some way the structure of the catalytic site. This structural effect could be repealed in the presence of Ca\(^{2+}\) (Matheson et al., 2006). The ability of ArpA to activate the neutrophil chemoattractant activity of IL-8 suggests that aeruginolysin could function as a provocative factor in the inflammatory response during a *P. aeruginosa* infection (Matheson et al., 2006). This assumption is confirmed by rat-model experiments demonstrating that *P. aeruginosa* proteases induce IL-8 accumulation with following neutrophil invasion (Kon et al., 1999).

Furthermore aeruginolysin is capable to degrade interleukin-6 very efficiently (Matheson et al., 2006). Cytokine IL-6 is an important factor in the host immune response during infection. Leu-Lys-aeruginolysin is more effective compared to the shorter variant in the degradation of IL-6. Another difference between both AprA forms is the efficiency of IL-6 intermediate degradation. The shorter form of aeruginolysin degrades IL-6 without any intermediates. It was suggested that the cytokine IL-6 takes part in a mechanism to regulate the inflammatory signaling activated by airway epithelial cells (Gomez et al., 2005). Aeruginolysin may hinder these IL-6 dependent regulatory systems. Potentially both types of aeruginolysin interfere with the cytokine circuits in lung (Matheson et al., 2006). However, the presence of Leu-Lys-aeruginolysin in patients sputum needs to be verified.

Purified aeruginolysin and elastase B are capable to degrade recombinant elafin (Guyot et al., 2010). The elafin protein is a 6 kDa distinct immune protein localized at many epithelial surfaces that is able to covalently bind extracellular matrix proteins via transglutamination. Elafin which belongs to the chelonianin family and functions as a protease inhibitor of the human neutrophil serine proteases, proteinase 3 and neutrophil elastase. When elafin is bound to fibronectin it still functions as an inhibitor and is able to protect fibronectin against...
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neutrophil elastase (Guyot et al., 2005). Elastase B is able to cleave the protease-binding loop that leads to the inactivation of the anti-neutrophil elastase activity of elafin. Degradation of elafin through aeruginolysin occurs at the N-terminal Lys6-Gly7 peptide bond. It was already shown that the first 1 – 8 amino acid residues of the N-terminus are not necessary for the antiprotease function of elafin (Francart et al., 1997; Tsunemi et al., 1996) but the elafin fragment absent from its N-terminus (Ser10-Gln57) is unable to bind purified fibronectin by transglutamination (Guyot et al., 2005). It was suggested that crosslinked elafin protects structural proteins against proteolysis caused by neutrophil serine proteases. Hence, the binding of elafin to fibronectin in vitro lead to the assumption that *P. aeruginosa* can regulate crosslinking of elafin to extracellular matrix proteins and therefore influence the protection against enhanced proteolysis caused by neutrophils (Guyot et al., 2010).

The role of *P. aeruginosa* alkaline protease in keratitis is still controversial. First it was reported that strains of *P. aeruginosa* PA103 deficient in alkaline protease production were not able to establish corneal infections and could not colonize traumatized cornea (Howe and Iglewski, 1984). Afterwards an in vivo infection study in mice showed that active alkaline protease was present in corneal tissues (Kernacki et al., 1997). In contrast Pillar and coworkers constructed alkaline protease-deficient mutants of *P. aeruginosa* to analyze the role of this protease in corneal infection and they were able to show that the alkaline protease has no influence on the ocular virulence of *P. aeruginosa* (Pillar et al., 2000). Because of this results it was concluded that alkaline protease is not essential for *P. aeruginosa* keratitis contrary to the former opinion. The role of secreted proteases in the pathogenesis of corneal disease was further examined by using proteases mutant strains of *P. aeruginosa* (Hobden, 2002). This study showed that the three proteases alkaline protease, LasA, and LasB are not essential for the establishment of the ocular virulence of *P. aeruginosa*. However, it was demonstrated that the alkaline protease seems to be a crucial mediator of virulence. This function of the alkaline protease depends on the location of *P. aeruginosa* within the cornea and on the presence of simultaneous elastinolytic activity (Hobden, 2002).

In a current study the two proteases LasB and aeruginolysin were expressed separately by the non-pathogenic organism *P. putida* using a rabbit model to determinde the specific virulence capability of *P. aeruginosa* proteases on bacterial keratitis (Thibodeaux et al., 2007). Elastase production by *P. putida* caused significant enhancement of corneal damage.
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The expression of alkaline protease caused limited corneal erosions. These results indicated that the potential of alkaline protease to cause corneal erosions during *P. aeruginosa* keratitis is limited.

**LasD**

The 23 kDa serine protease LasD (Park and Galloway, 1995) is a second staphylolytic protease of *P. aeruginosa*. It shows similarity to the LasA protease of *P. aeruginosa* and is therefore able to cause the lysis of staphylococcal cells (Park and Galloway, 1995). Staphylolytic activity of LasD, as well as those of LasA, results from the ability to cleave the pentaglycine motif of *Staphylococcus* murein. It was shown *in vitro* that interaction of LasD and LasA occurs through specific cleavage of proLasA by LasD to generate the active LasA protease. The results of this study indicate that LasA processing occurs through the specific action of LasD (Park and Galloway, 1998).

LasD is secreted via the Xcp (type II) pathway (Braun et al., 1998). Typical for the secretion of proteases via the type II pathway is the translocation of precursors. It was shown that LasD, like the LasA protease, appears to be secreted as a proenzyme (Braun et al., 1998). The 23 kDa mature form is located at the N-terminal domain and, therefore, LasD possesses a putative C-terminal propeptide (Braun et al., 1998). Processing of pro-LasD occurs after translocation across the outer membrane which is another similarity of LasD and LasA. Elastase B is essential for the processing of pro-LasD into the 23 kDa form (Braun et al., 1998).

**PasP**

The *P. aeruginosa* small protease (PasP) was originally identified and characterized in the *P. aeruginosa* strain PA103 by Marquart and coworkers (Marquart et al., 2005). PasP is a secreted protease and was found to possess a molecular weight of 18.5 kDa. It was assumed that the protease is secreted via the Xcp (type II) secretion pathway because of the identification of a N-terminal putative signal sequence for Sec-dependent translocation suggesting a two step secretion process (Marquart et al., 2005).
The \textit{pasP} gene was demonstrated to be commonly found in \textit{P. aeruginosa} isolates which lead to the assumption that the gene encoding for PasP is conserved among \textit{P. aeruginosa} strains (Tang et al., 2009). Tang and coworkers were able to show that the gene encoding for PasP is commonly found in \textit{P. aeruginosa} isolates and that the secretion of the PasP protease into \textit{P. aeruginosa} culture supernatants is present for several \textit{P. aeruginosa} strains that differ in geographic origins and organs of the infection from which they were isolated (Tang et al., 2009). This widespread presence of PasP indicates that this protease, like protease IV (Caballero et al., 2004), is a protease with high dispersion in \textit{P. aeruginosa} isolates which may suggest a prominent and general role of the enzyme during infection. It was shown that the size of the PasP protease is the same in the periplasm as is found in the supernatant (Tang et al., 2009). Hence PasP is a protease that is not produced as a larger proenzyme with following processing resulting in a smaller active form. The enzymatic active variant of PasP was only present in supernatants after secretion (Tang et al., 2009). Moreover the production of PasP appears to be regulated by the quorum sensing systems (Arevalo-Ferro et al., 2003). PasP is able to cleave type I and IV collagens (Tang et al., 2009), causes corneal erosions (Marquart et al., 2005; Tang et al., 2009) and it was shown that PasP is made during infections in CF patients (Uritchard et al., 2008). Furthermore Tang and coworkers tested the presence of PasP in ocular isolates, nonocular clinical isolates and laboratory strains suggesting that PasP could play a role in several \textit{Pseudomonas} infections not only in keratitis. It was demonstrated that the injection of active recombinant PasP into the cornea induce an inflammatory response by an unknown mechanism. One part of this inflammatory response is the migration of polymorphonuclear leukocytes into the limbus, the corneal stroma and the anterior chamber (Tang et al., 2009).

\textbf{Aminopeptidase}

In general aminopeptidases are exopeptidases that selectively release N-terminal amino acid residues from protein-substrates (Gonzales and Robert-Baudouy, 1996). The amount of information about secreted exopeptidases of \textit{P. aeruginosa} is rather limited at present. The aminopeptidase \textit{PaAP} of \textit{P. aeruginosa} is one of these extracellular proteases (Cahan et al., 2001) that is probably regulated in part by the \textit{las} quorum-sensing system (Nouwens et al., 2003). The \textit{AP}_{28} form shares high homology and possesses similar properties e.g. to the
aminopeptidases SgAP (*Streptomyces griseus*) and VpAP (*Vibrio proteolyticus*) (Cahan et al., 2001). Due to the homology to these other aminopeptidases, PaAP was categorized in the same family of Zn$^{2+}$-dependent metallopeptidases (Cahan et al., 2001).

The deduced amino acid sequence of PaAP reveals a 58 kDa putative aminopeptidase. It was shown that PaAP occurs in two active forms. On the one hand in a 56 kDa form (AP$_{56}$) and on the other hand in a 28 kDa form (AP$_{28}$) (Cahan et al., 2001). The amino acid sequence of the *paAP* gene product shows that the active 28 kDa domain is located in the C-terminal part of AP$_{56}$.

The characterization of PaAP demonstrated that PaAP is a heat stable protein with an pH optimum for AP$_{28}$ hydrolysis of 8.5 using Leu-pNA as a substrate (Cahan et al., 2001). It is suggested that PaAP is secreted by the Xcp (type II) secretion pathway because of the identification of a putative signal peptide in PaAP and the fact that AP$_{58}$ is not detectable in the culture supernatants of an *xcp* mutant strain of *P. aeruginosa* (Braun et al., 1998; Cahan et al., 2001). PaAP is not extensively processed under normal growth conditions like most of the other major secreted proteases of *P. aeruginosa* like LasA, LasB or protease IV.

The combination of the proteases elastase B and alkaline protease or the alkaline protease alone cause limited proteolysis of AP$_{58}$. A short N-terminal sequence is removed resulting in the conversion from AP$_{58}$ to AP$_{56}$ but no further processing takes place. It was assumed that the short N-terminal prosequence could function as an inhibitor to the enzyme so that its removal results in the activation of the putatively inactive AP$_{58}$ (Cahan et al., 2001). The function of the long N-terminal sequence of AP$_{56}$ which is not present in the shorter AP$_{28}$ form is unknown so far. It was assumed that this sequence is essential for the control of proteolytic activity of PaAP (Cahan et al., 2001).

The determination of the physiological role of PaAP needs further investigation. It has been supposed that the role of secreted aminopeptidases in general is the release of free amino acids. These amino acids that result from the cleavage of peptide-substrates could be used for propagation, cell growth and nutrition (Gonzales and Robert-Baudouy, 1996; Maeda and Yamamoto, 1996). Furthermore it is imaginable that the PaAP activity complements the activity of the four major endopeptidases secreted by *P. aeruginosa* (Cahan et al., 2001). Such interactions between exopeptidases and endopeptidases have already been reported (Wagner et al., 1972). Maybe the combined action of PaAP and the other secreted endopeptidases lead to the liberation of free amino acids (Cahan et al., 2001).
PaAP was shown to be a major component of outer membrane vesicles released by *P. aeruginosa* (Bauman and Kuehn, 2006). Such outer membrane vesicles have been observed in many other Gram-negative pathogens (Beveridge, 1999; Kuehn, 2005) and *P. aeruginosa* is one of them. As already mentioned, PaAP is thought to be secreted via the Xcp secretion pathway. The finding that PaAP is the major component of outer membrane vesicles could relativize this opinion. Because of the low size of *P. aeruginosa* vesicles and therefore the difficulty to get vesicle-free supernatants it is not clear whether PaAP is secreted by the bacterium or released within vesicles or maybe both (Bauman and Kuehn, 2006). Quantification, purification and characterization of *P. aeruginosa* outer membrane vesicles illustrated that PaAP was significantly enriched in vesicles from cultures of CF isolates (Bauman and Kuehn, 2006). The yield of *P. aeruginosa* vesicles seems to be strain independent but the vesicle composition seems to be addicted to the host environment (Bauman and Kuehn, 2006). The release of vesicles results in a significant activation of IL-8 proinflammatory response in the epithelial cells of lung (Bauman and Kuehn, 2006). The physiological roles of *P. aeruginosa* vesicles during infection and the role of PaAP within this vesicle system have to be further determined.
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"As the fractal grows a more distinct form appears."
CHAPTER 5
Results and discussion

CTP target identification: Secretomics

This chapter is based on:

Chapter summary

Previous observation by Rosenau showed that ΔctpA and Δprc inactivated strains of *Pseudomonas aeruginosa* secreted less of the protein lipase LipA (Rosenau and Jaeger, 2004). Research on CTP defective strains of other Gram negative bacteria such as *Brucella suis* and *Burkholderia mallei* showed to be less virulent.

In the search of novel antibiotargets in *P. aeruginosa*, these observations led to further investigate the secretome of the CTP inactivated mutants. Therefore 2D gel electrophoresis analysis was executed on the supernatant of ΔctpA and Δprc of *P. aeruginosa* cultures.

The 2D gel electrophoresis analysis show that several protein were less secreted in the culture supernatant. Among these protein were major virulence factors such as Pseudolysin, Staphylolysin, Alkaline protease, Protease IV and the Aminopeptidase PepB. In order the validate these secretome observations enzyme specific assays were performed on these virulence factors.

The results of these enzyme specific assays confirmed the decreased secretion of virulence factors by the 2D gel electrophoresis. The assay showed that the virulence factors were indeed secreted in much lower amounts. One of the major virulence factors Pseudolysin was shown to be secreted a magnitude 10 less in both ΔctpA and Δprc.

The results show that the CTPs in *P. aeruginosa* effect the secretion of proteins among which are major virulence factors. Because the decreased virulence factors are secreted by different systems it seems plausible that the CTP effect the secretion on a more basal level.

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CTP target identification: Secretomics

Epidemic increases in bacterial multidrug resistance and the occurrence of truly pan-resistant Gram-negative pathogens (e.g., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*) are major concerns, novel antibiotics that interrupt the host-pathogen interaction are urgently needed (Payne, 2008).

The Gram-negative pathogens *Brucella suis* and *Burkholderia mallei* were less virulent, when CtpA was inactivated (Bandara et al., 2008; Bandara et al., 2005) and CtpA from *Chlamydia trachomatis* was shown to interfere with the NF-κB pathway of human immune response (Mitchell and Minnick, 1997) suggesting yet unknown roles for each of these proteases in virulence. Recently Tsp inactivation in a pathogenic strain of *E.coli* showed a decreased ability to cause bacteremia (Wang et al., 2012).

What the physiological function of CTPs are is still unknown in order to explain the decreased in virulence (Hoge et al., 2011). In perspective of the identification of a novel antibiotic target the CTP in *Pseudomonas aeruginosa* would be interesting as it seems to reduce virulence.

Inactive CTP deletion mutants were constructed by the group of Rosenau. The ΔctpA mutant showed a decreased extracellular levels of secreted lipase LipA in *P.aeruginosa* (Rosenau and Jaeger, 2004). This fact lead us to further investigate the secretion of proteins in these CTP defective mutants. The effect of secretion of in these mutants was examined by analysis of the secretome by 2D gel electrophoresis. Spot intensities were analyzed and compared. Spot could be identified by MALDI-TOF peptide fingerprinting. The 2D gel secretomes of the wildtype *P.aeruginosa* strain and ΔctpA are depicted in figure 5.1 and of Δprc in figure 5.2.
Figure 5.1: Secretome of 16 hr. cultures of *P. aeruginosa* wildtype (A) and mutant ΔctpA (B). Spots were identified by MALDI-TOF peptide fingerprinting: Alkaline protease (AprA), Pseudolysine (Ply), Staphylolysine (Sly) Protease IV (PIV) and Aminopeptidase PepB (PepB).
Figure 5.2: Secretome of 16 hr. cultures of *P. aeruginosa* wildtype (A) and mutant ∆prc (B). Spots were identified by MALDI-TOF peptide fingerprinting: Alkaline protease (AprA), Pseudolysine (Ply), Staphylolysine (Sly) Protease IV (PIV) and Aminopeptidase PepB (PepB).
Figure 5.3. A) Secretome protein expression profiles of the CTP mutants of *P. aeruginosa* PAO1 ΔctpA and ΔPrc compared to wild type. Protein levels of supernatants are shown after 16 h growth analysed by 2D gel electrophoresis (n = 4 for each strain). Standard deviations are represented by the error bars. B) Relative enzyme activities of selected virulence related proteases of the CTP mutant strains compared to wild type in the supernatant after 16 h growth. Standard deviations are represented by the error bars.
The spots that were identified as known virulence factors were further examined. The virulence factors identified spots where: Pseudolysin, Staphylolysin, Protease IV, Alkaline protease and Aminoprotease PepB. Figure 5.3A gives an detail relative protein secretion profile of these virulence factors comparing wilttype with the CPT mutants ΔctpA and Δprc based on the secretome analysis.

Both mutant strains ΔctpA and Δprc showed an decreased amount of secreted virulence factors. ΔctpA showed an overall reduction of secreted virulence factors of about 1/3 and Δprc more than 1/2 based on the secretome 2D gel electrophoresis.

In order to confirm these results enzyme specific activity assay were conducted for these virulence factors in the supernatant of the cultures. Figure 5.3B shows the results of these enzyme assays. The overall activity assay of the secreted virulence factors confirm the 2D gel electrophorees results. All activities are reduced in the both CTP defective mutants. The actual relative activities seems to be even more attenuated than the 2D gel results suggest. The relative Pseudolysin activity was decreased to 10% in ΔctpA and 13% in Δprc, thus a 10x magnitude less. Pseudolysin also named Elastase B is one of the most well known secreted virulence factor of P.aeruginosa. Pseudolysin is also one of the most abounded protein secreted by P.aeruginosa as can be seen in figure 5.1/5.2. No activity of Protease IV could be detected in the supernatant of the ΔctpA and Δprc strains. Although Protease IV spots were detected and indentified with both mutants in the 2D gel electrophoresis the activity assay suggests that the Protease IV secreted in the supernatant is not active.

Alkaline protease activity was <25% in the ΔctpA strain and 38% in the Δprc strain. Staphylolysin also named Elastase A activity was determined by examining the ability of the supernatant to lyse a Staphylococcus aureus strain. The supernatant of the ΔctpA strain showed a relative activity of 21% and 27% for the ΔctpA strain.

The last virulence factor tested was the Aminopeptidase PepB. Compared to the wildtype, ΔctpA had a relative aminopeptidase acitivity of 10% and the Δprc strain 13%.
Chapter 5

The 2D gel electrophoresis secretome analysis clearly showed a reduced amount of secreted virulence factors present in the supernatants of *P. aeruginosa* in the CTP mutants. These observations were confirmed by the enzyme specific assays. Although the magnitude of activity reduction in the supernatant was much lower in the specific assay than was in het 2D gels analysis. A lower enzyme activity can be the result of less enzyme present or also due to a lower activity of the enzyme. The 2D gel electrophoresis analyses is less sensitive for secretion differences. Although for Protease IV no specific activity could be measured in the supernatant of the CTP mutants. Protease IV spots could be identified in the 2D gel secretome analysis. Therefore it is more plausible that the protease IV is secreted in lower amount by the CTP mutants but not in its active form.

**Chapter Discussion**

The result based on the 2D gel electrophoresis and the subsequent enzyme specific assay show that both CTP mutants are defective in the secretion of virulence factors. Based on these experiments a possible mechanism could be postulated why other CTP defective mutant strains were less virulent. CTP is clearly necessary in *P. aeruginosa* for a functional secretion system. This could depend on the different secretion systems present on bacteria. Alkaline protease is virulence factor that is secreted by a type I secretion system (Guzzo et al., 1991). The other virulence factors Pseudolysin and Staphylolysin are secreted by a type II secretion system. The secretion pathways of Protease IV and the Aminopeptidase PepB are unknown until now. Because of the different secretion pathways of Alkaline protease and both Pseudolysin and Staphylolysin it seems to be unlikely that CTP plays a role in one specific secretion pathway but that is influence secretion on a more basal level. These results suggest that the CTP defective strains in *Pseudomonas aeruginosa* could be less virulent because of they are attenuated in the secretion of virulence factors. This hypothesis was tested in the next chapter.
References


"With subsequent drawings of the fractal curve, sudden changes may appear."

IAN MALCOLM

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff
CHAPTER 6
Results and discussion

CTP target validation: infection models

This chapter is partly based on:

Chapter summary

The $\Delta$ctpA and $\Delta$prc mutant strains of \textit{P.aeruginosa} were shown to be defective in the secretion of virulence factors. This would suggest that the strains are also less virulence. The virulence of these CTP mutant strains were investigated in several infection models.

Both $\Delta$ctpA and $\Delta$prc were less pathogenic in the \textit{Arabidopsis thaliana} plant infection model as shown by a reduced bacterial growth in the leaves.

The CTP strains were also tested in two different fly \textit{Drosophila melanogaster} infection model. $\Delta$ctpA and $\Delta$prc were less virulent in a feeding assay as median survival times were prolonged. Virulence was also assessed in a second assay as \textit{D.melanogaster} flies were pricked with bacteria causing a systemic infection. $\Delta$ctpA showed a significant prolonged survival time but $\Delta$prc showed only a marginal difference compared to the wild type.

$\Delta$ctpA and $\Delta$prc were also shown to be less virulence in the nematode \textit{Caenorhabditis elegans} slow killing assay. Both CTP mutants showed a significant lower killing of the nematodes than the wild type \textit{P.aeruginosa} strain.

Larvae of the Greater wax moth, \textit{Galleria mellonella} are very sensitive for systemic infection caused by \textit{P.aeruginosa}. LD$_{50}$ values were determined after injecting different bacterial doses. Both $\Delta$ctpA and $\Delta$prc strains had a larger LD$_{50}$ than the wild type which was significant for $\Delta$ctpA but not for $\Delta$prc. In a second assay the extracellular fraction of the strain were injected into the larvae of the moth to assess mortality. The sterile extracellular fraction of the wild type was lethal for the larvae. The $\Delta$ctpA and $\Delta$prc showed a longer mean survival times when compared to the wild type.

The results of these different infection models show that CTP defective strains are less pathogenic and defective in virulence. The decreased ability to secrete virulence factors of the CTP inactive \textit{P.aeruginosa} strains may well explain this reduced pathogenesis.

The results of this chapter were obtained in collaboration with:

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CTP target validation: Infection Models

In chapter 5 it was shown that the CTP defective \textit{P. aeruginosa} mutants \textit{ΔctpA} and \textit{Δprc} were disrupted in the secretion of proteins among which major virulence factors. This led to the hypothesis that the \textit{ΔctpA} and \textit{Δprc} \textit{P. aeruginosa} strains are less virulence. In order to test this hypothesis the CTP defective strains were evaluated in different infection models. Four different infection models were tested: Thale cress (\textit{Arabidopsis thaliana}), Fruit fly (\textit{Drosophila melanogaster}), \textit{Caenorhabditis elegans} and the Greater Wax Moth (\textit{Galleria Mellonella}). All models are well respected infection models for evaluating the pathogenesis of \textit{Pseudomonas aeruginosa} (Jander et al., 2000).

Other CTP inactivated bacterial mutants have shown to be involved in pathogenesis. Recently inactivation of CTP was shown to influence pathogenesis of several Gram-negative bacteria. In a \textit{ΔctpA} strain of \textit{Brucella suis} declined CFU survival was measured in a macrophage cell line. Subsequent infection analysis in mice after intraperitoneal inoculation, CFU survival in spleen showed that the \textit{ΔctpA} strain could not be detected after nine weeks post inoculation were as the wildtype was detected. This could indicate that CtpA was necessary of intracellular survival of the bacterium. Mice exposed to the \textit{ΔctpA} strain protected them of \textit{B. abortus} and \textit{B. suis} infection (Bandara et al., 2005). The virulence attenuated \textit{ΔctpA} strain of \textit{Brucella suis} is therefore in development as a vaccine against \textit{Brucella} species (Bandara et al., 2007). A \textit{ctpA} defective strain of \textit{Burkholderia mallei} was less able to survive in macrophages. The \textit{ΔctpA} strain of \textit{B. mallei} also showed reduced virulence in mice. Mice exposed to the \textit{ΔctpA} strain were partly protected against a \textit{B. mallei} infection (Bandara et al., 2008). A study performed on \textit{Chlamydia trachomatis} identified CtpA as a protease that was able to evade the immune NF-κB pathway by processing the p65 protein. This would enable \textit{C. trachomatis} to survive intracellular (Lad et al., 2007).
**Arabidopsis thaliana infection model:**

The ΔctpA and Δprc *P. aeruginosa* strains were tested in the *Arabidosis thaliana* infection model. Figure 6.1 shows the results of this model and table 1 gives the results in numbers. CFU counts were measured at day 2 and 4 after infection. After two days the bacterial load of the leafs of the plants that were infected had increased with log 3 for both the wild type and the two CTP defective strains. At day 5 the bacterial load increased further in the plants infected with the wild type strain to 1.6 x10^7 CFU ml^-1. The bacterial load of the two CTP defective strains decreased after two days. The bacterial load at day 4 of strain ΔctpA is 1.7 x10^6 CFU l^-1 this is about 10 fold less than the wild type. The bacterial load of the Δprc strain is 5.3 x10^5 CFU ml^-1 which is about 30 fold less than the wild type. When comparing the bacterial load at day 4 the difference between the wild type is statistical significant less (p < 0.05) than for both CTP defective strains ΔctpA (p = 0.027) and Δprc (p = 0.025). Mutually the bacterial load of the Δprc strain is about 1.5 fold lower than the ΔctpA strain which is not significant (p= 0.054).

The *Arabidosis thaliana* infection model is a well recognized model for evaluating pathogenesis of *P. aeruginosa* (Rahme et al., 1997). Several virulence factors have been identified using the *Arabidosis* model such as MvfR. The *Arabidosis* model gives also a good correlation of virulence with the mouse burn model (Rahme et al., 2000). Evaluation of a toxA mutant of *P. aeruginosa* PA14 in the *Arabidosis thaliana* model gave a decrease in bacterial load of 2.6 x10^7 in the wild type to 1.5 x10^6 in the toxA defective mutant. A decrease of factor 17. In the corresponding mouse burn model a reduction in mortality was observed from 77% in the wild type to 40% with the toxA mutant. Endotoxine A is besides Elastase B one of *P. aeruginosa* most important virulence factors. The bacterial growth reduction of the CTP mutant strains are comparable with the well known ΔtoxA mutant. These data suggests that both CTP inactive strains are less virulence in the *Arabididosis* model when comparing their log reduction with a toxA mutant . The Δprc mutant seems to be the less pathogenic variant when comparing with the ΔctpA strain. Although the wild type strain is different (PA14 versus PAO1) the observed reductions have the same dimensions. We can explain the observed reduction in bacterial load by the fact that CTP defective strains as shown in chapter 5 are defective in virulence factor secretion.
Figure 6.1: *Arabidopsis thaliana* leaf infection model: 4 plants x 4 leaves were infected with $10^3$ CFU ml$^{-1}$ bacteria of *P. aeruginosa* strains wild type, ΔctpA and Δprc. CFUs were measured at days 0, 2 and 4 after infection.

Table 6.1: *Arabidopsis thaliana* leaf infection model CFU count.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>ΔctpA</th>
<th>Δprc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU ml$^{-1}$</td>
<td>SD</td>
<td>CFU ml$^{-1}$</td>
<td>SD</td>
</tr>
<tr>
<td>Day 0  (n=2)</td>
<td>4.5 x10$^2$</td>
<td>0.0 x10$^2$</td>
<td>1.3 x10$^3$</td>
</tr>
<tr>
<td>Day 2  (n=4)</td>
<td>4.2 x10$^4$</td>
<td>3.8 x10$^6$</td>
<td>3.2 x10$^6$</td>
</tr>
<tr>
<td>Day 4  (n=4)</td>
<td>1.6 x10$^7$</td>
<td>8.1 x10$^6$</td>
<td>1.7 x10$^6$</td>
</tr>
</tbody>
</table>

*p value between CTP defective strain and the wild type. † p value between CTP defective strains mutually.
**Drosophila melanogaster infection model**

*Drosophila melanogaster* is a well respected infection model for evaluation several bacterial, fungal and virus pathogenesis (Panayidou et al., 2014). *D. melanogaster* is also a good infection model specific for *P. aeruginosa* (Apidianakis and Rahme, 2009). Pathogenesis of *P. aeruginosa* can be evaluated with two different *Drosophila* infection models. The first model is the *P. aeruginosa* feeding assay, the second model is the *Drosophila* pricking model. Because *P. aeruginosa* is an opportunistic pathogen in humans, the bacterium only establishes infection when the host own defense mechanisms are weakened. The pricking assay, by infecting the *Drosophila* fly with a needle, models this. The feeding experiment will simulate a infection of the intestine of the fly (Apidianakis and Rahme, 2009).

**Figure 6.2:** Illustrative picture of the *Drosophila melanogaster* feeding assay at day 8 after infection. Left container: flies fed on *P. aeruginosa* wild type. Right container: flies fed on *P. aeruginosa ΔctpA*. 
**Drosophila melanogaster infection model – feeding assay**

Both ΔctpA and Δprc mutant strains were tested in the *D.melanogaster* feeding assay. Flies were fed during 12 days on a sucrose solution containing a defined amount of bacterial culture. Figure 6.2 gives an illustrative picture of the flies being killed when fed on *P.aeruginosa*. In the left container flies were grown on the *P.aeruginosa* wild type strain and on the right on the ΔctpA strain at day after infection. Although it is difficult to see which flies are death, most flies that are alive stick to the side of the container.

The results of the Kaplan-Meier survival curve are depicted in figure 6.3. The flies that were grown on the *P.aeruginosa* wild type strain were all dead after 10 days. Flies that were fed on the CTP deficient mutants of *P.aeruginosa* were dead after 12 days. The Kaplan-Meier survival curves of both mutant strains lies well above the wild type. The killing of the flies is faster when grown on the wild type strain. Table 6.2 shows the corresponding median survival times of the Kaplan-Meier survival curves. The median survival time of the flies fed on the wild type strains was 6 days (CI95%: 4.9-7.1). The CTP defective mutants mean survival times were for both ΔctpA and Δprc 10.0 days. Flies lived significant longer on the CTP defective strains when compared to the wild type *P.aeruginosa*. There was no difference in survival times between the two CTP defective strains.
**Drosophila melanogaster feeding assay**

Kaplan-Meier survival curve

![Kaplan-Meier survival curve](image)

Figure 6.3: Kaplan-Meier survival curve of *Drosophila melanogaster* feeding assay of CTP mutants ∆ctpA and ∆prc in comparison to wild type *P. aeruginosa* (n=60 per strain).

Table 6.2: Mean survival times of *Drosophila melanogaster* in the feeding assay of CTP defective *P. aeruginosa* strains.

<table>
<thead>
<tr>
<th>Strains <em>P. aeruginosa</em></th>
<th>Median survival time (days)</th>
<th>CI95% (days)</th>
<th>P-value Log Rank (Mantel-Cox)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>6.0</td>
<td>4.9-7.1</td>
<td></td>
</tr>
<tr>
<td>∆ctpA</td>
<td>10.0</td>
<td>9.6-10.4</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>∆prc</td>
<td>10.0</td>
<td>9.7-10.3</td>
<td>&lt; 0.000</td>
</tr>
</tbody>
</table>
**Drosophila melanogaster infection model - needle pricking assay**

The *D. melanogaster* needle pricking assay has been tested for the CTP defective strains by infecting the flies by pricking the abdomen with a needle dipped in a bacterial suspension (about 100 CFU per pricking). Figure 6.4 shows the resulting Kaplan-Meier survival curve and median survival times are presented in table 6.2. In the graph can be seen that the killing is fastest with the flies infected with the wild type *P. aeruginosa* strain. Although the deviations between the different curves are not big. In the beginning there seems no difference in the killing rate between the wild type and the CTP defective strains. As the time of infection increases less flies are dying from the CTP defective strains. The median survival time of the flies infected with the wild type strain is 29.0 hours. The median survival time of the ΔctpA and Δprc strains are respectively 32.0 and 31.0 hours. The difference between the Kaplan-Meier curves is significant for the ΔctpA versus the wild type strain (p= 0.02) but not for the Δprc when comparing with the wild type (p=0.16). Despite the significance of the deviation there is no great difference in survival between the flies infected with the wild type and the two CTP defective strains.

This assay gives a different result as seen in the *D. melanogaster* feeding assay. In the feeding assay the virulence of the wild type was more clearly shown than in the needle pricking assay, even if the same organism is involved. The way the infection was established within the organism depended on the assay. With the needle pricking assay all natural defense mechanisms are bypassed and the flies are directly systemically infected. This is a whole different mechanism when compared to the feeding assay in which the flies are infected via food intake. Before causing a systemic infection *P. aeruginosa* has first be able to tackle the defense mechanisms in the gastrointestinal tract of the fly and afterwards enter the body. As shown in chapter 5 the CTP inactivated strains are defective in secretion of several virulence factors. Based on these results and the infection experiments with *Drosophila melanogaster* it seems that these secretion factors could be important for starting a infection in the intestine of the flies. Physical barriers and the antimicrobial peptidase protect the fly from invasive pathogens. Before *P. aeruginosa* is able to establish an infection it has to penetrate the exoskeleton or the intestinal epithelium of the fly. Once inside the systemic body of the fly there seems to be no great difference in virulence between a wild type infection or a defect in one of the two CTPs.
Figure 6.4: Kaplan-Meier survival curve of *Drosophila melanogaster* needle pricking assay of CTP mutants ∆ctpA and ∆prc in comparison to wild type *P. aeruginosa* (n=80 per strain). Flies were pricked by an needle with about 100 CFUs and mortality was monitored for 48 hours at 25 °C.

Table 6.2: Mean survival times of *Drosophila melanogaster* in the needle pricking assay of CTP defective *P. aeruginosa* strains.

<table>
<thead>
<tr>
<th>Strains P. aeruginosa</th>
<th>Median survival time (hours)</th>
<th>CI95% (hours)</th>
<th>P-value Log Rank (Mantel-Cox)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>29.0</td>
<td>27.5-30.4</td>
<td></td>
</tr>
<tr>
<td>∆ctpA</td>
<td>32.0</td>
<td>22.3-41.7</td>
<td>0.02</td>
</tr>
<tr>
<td>∆prc</td>
<td>31.0</td>
<td>26.7-35.4</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Caenorhabditis elegans infection model

Caenorhabditis elegans is one of the simplest invertebrate models for investigation the virulence of different bacteria. C. elegans can be used in different assays of assessing the virulence of P. aeruginosa (Papaioannou et al., 2013). The most widely used assay is the C. elegans slow killing assay. In this model P. aeruginosa is grown on a minimal medium from which the C. elegans feeds itself on a lawn of Escherichia coli bacteria. This model is assessed over several days in which the bacterium establishes an infection in the intestine of the nematode. Figure 6.4 shows the results of the C. elegans infection model when the nematode is infected with P. aeruginosa wild type in comparison to the ΔctpA and Δprc strains. The assay was replicated four times. Per strain 200 nematodes were assessed.

It is clearly visible that the C. elegans nematodes are dying faster when infected with the wild type P. aeruginosa strain. Nematodes start to die 4 days after infection and about 50% of the nematodes have died after 7 days. When C. elegans is infected with the CTP defective strains more than 90% of the nematodes is still alive after 7 days. Table 6.3 represents the mean survival times established in this infection model. The mean survival time of C. elegans when infected with the wild type is 6.1 days. The mean survival times of the ΔctpA and Δprc strains could not be determined because of the less death events occurred with these strains but the mean survival time lies beyond 8 days. The difference in mean survival times between the wild type and the CTP defective strains were significant.
Figure 6.4: Kaplan-Meier survival curve of the *Caenorhabditis elegans* slow killing assay of CTP mutants ΔctpA and Δprc in comparison to wild type *P. aeruginosa* (n=200 per strain). *C. elegans* L4 nematodes were grown on a lawn of *E. coli* infected with the test strains for 1 week at 24°C. Mortality was monitored every 12 hours.

Table 6.3: Mean survival times of *Caenorhabditis elegans* in the slow killing assay of CTP defective *P. aeruginosa* strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mean survival time (days)</th>
<th>CI95% (days)</th>
<th>P-value Log Rank (Mantel-Cox)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>6.1</td>
<td>6.0-6.2</td>
<td></td>
</tr>
<tr>
<td>ΔctpA</td>
<td>&gt; 8*</td>
<td>ND*</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>Δprc</td>
<td>&gt; 8*</td>
<td>ND*</td>
<td>&lt;0.000</td>
</tr>
</tbody>
</table>

* mean survival time could not be determined because not enough death events occurred.
**Galleria mellonella infection model**

The Great wax moth or *Galleria mellonella* is very sensitive for systemic *P.aeruginosa* infection and is used extensively for evaluating its virulence. A big advantage of this model is that the larvae can be handled well because of their size (250 mg). This opens the possibility to be able to inject a defined volume of a solution into the organism such as necessary to estimate LD$_{50}$ values. Besides that has the infection model a good correlation with pathogenesis of *P.aeruginosa* in mice (Jander et al., 2000).

**Galleria mellonella infection model-injection assay**

Therefore the virulence of the ∆ctpA and ∆prc strains were tested in the *Galleria mellonella* infection model. Hereeto larvae of the moth were injected with different bacterial CFU doses. Figure 6.5 shows the survival probability of the differed bacterial doses injected into the larvae. The figure gives an overview of the killing rate with the increasing bacterial doses of three in depended experiments. It can be seen that the killing rate is smaller with the ∆prc strain and also with the ∆ctpA strain although this difference is less evident when compared to the wild type strain.

Based on these data a 50% lethal dose could be estimated as presented in table 6.4. The LD$_{50}$ was estimated based on the killing patron of 180 larvea per strain. ∆ctpA had an LD$_{50}$ value of 12.1 CFU which was 6,7 fold higher than the wild type strain with a LD$_{50}$ of 1.8 CFU. The difference was statistical significant. The ∆prc had a 2 fold higher LD$_{50}$ value of 3,6 CFU as compared to the wild type strain although not significant. There was no difference in attenuation of virulence between the both CTP mutants. Overall it is remarkable how sensitive *G.mellonella* is for systemic infection as a few bacteria is enough to kill the moth. These results indicate that both CTP inactivated strains are attenuated in its virulence as shown with other infection models.
Figure 6.5: Graphical representation of the killing of *Galleria mellonella* larvae after injection of different bacterial dosis (10^6, 10^4, 10^2, 10^1, 10^0 and 10^-1 CFU/injection) of CTP mutants ΔctpA and Δprc in comparison to wild type *P.aeruginosa*. 3x 10 larvae were injected per doses (total 180 larvea per strain). Mortality was monitored after 3 days incubated at 24°C.

Table 6.4: LD₅₀ estimation (Probit analysis) of *P.aeruginosa* mutant strains ΔctpA, ΔPrC and wild type in *Galleria mellonella* larva (n=3x 10 larvea per dilution: 10⁶, 10⁴, 10², 10¹, 10⁰ and 10⁻¹; total 180 larvea per strain) after 3 days at 24 °C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD50</th>
<th>95% CI</th>
<th>Virulence decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.8</td>
<td>1.4-2.4</td>
<td></td>
</tr>
<tr>
<td>ΔctpA</td>
<td>12.1</td>
<td>2.8-33.1</td>
<td>6.7x</td>
</tr>
<tr>
<td>Δprc</td>
<td>3.6</td>
<td>2.3-4.9</td>
<td>2x</td>
</tr>
</tbody>
</table>
**Galleria mellonella infection model – supernatant injection assay**

As shown in chapter 5 the CTP inactive strains are defective in the secretion of several virulence factors. There is no significant difference between the extracellular fraction of ΔctpA and Δprc which could explain difference in virulence of the two mutants in *G.mellonella*. In order to investigate which role the secreted proteins play in the virulence of *G.mellonella*, extracellular fractions of the CTPs inactivated strains were tested in the moth larvae.

20 µl of an extracellular fraction was injected into 3x10 larvae per strain and mortality of the larvae were scored for 6 days. Figure 6.6 shows the calculated Kaplan-Meier survival curve and table 6.5 presents the mean survival times. The first thing that stands out is that sterile supernatant of *P.aeruginosa* is lethal for the larvae at noticed with the wild type strain (see fig. 6.6). After 4 days all the larvae were dead as result of the injected supernatant. As a control 3x 5 larvea were injected with LB medium. Al injected larvae were alive after 6 days. The injected extracellular fractions were proven to be sterile as no growth was observed after incubation for 2 days in LB medium. The larvae with the extracellular fraction of the ΔctpA strain showed a significant longer mean survival time of 5.0 days (CI 95%: 4.6-5.4) as compared to the wild type strain with a mean survival time of 3.8 days (CI 95%: 3.4-4.4). For the Δprc strain with a mean survival time of 4.1 (CI 95%: 3.6-4.9) no signification difference to the wild type could be shown.
Figure 6.6: Kaplan-Meier survival curve of *Galleria mellonella* supernatant injection assay of CTP mutants ΔctpA and Δprc in comparison to wild type *P. aeruginosa* (n= per strain). Flies were pricked by an needle with about 100 CFUs and mortality was monitored for 48 hours at 25 °C.

Table 6.5: Mean survival times of *Galleria mellonella* in the supernatant injection assay of the CTP defective *P. aeruginosa* strains ΔctpA and Δprc.

<table>
<thead>
<tr>
<th>Strains <em>P. aeruginosa</em></th>
<th>Mean survival time (days)</th>
<th>CI95% (days)</th>
<th>P-value Log Rank (Mantel-Cox)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>3.8</td>
<td>3.4-4.4</td>
<td></td>
</tr>
<tr>
<td>ΔctpA</td>
<td>5.0</td>
<td>4.6-5.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Δprc</td>
<td>4.1</td>
<td>3.6-4.9</td>
<td>0.09</td>
</tr>
</tbody>
</table>
The injected extracellular fractions were grown and harvested on the same conditions as the secretome analysis was conducted. It is important to verify that the fractions were absolutely sterile because of the high pathogenicity of *P. aeruginosa* for this moth with a LD$_{50}$ of a few CFU as shown before. The extracellular fraction of the ΔctpA strain was clearly less virulent than the wild type strain. This confirms our hypothesis that the defect in the secretion of virulence factors makes *P. aeruginosa* less pathogenic. But even without the bacterium itself the supernatant of *P. aeruginosa* is lethal. The extracellular fraction of the Δprc strain did show only a marginal difference as compared to the wild type. A remarkable difference between the LD$_{50}$ determination and the injection of the extracellular fraction is that Δprc strain has the highest LD$_{50}$ and is therefore less virulent than the ΔctpA strain. But virulence of the Δprc strain was less attenuated compared to the ΔctpA strain when injecting the extracellular fraction. As an explanation there could be difference in a secreted virulence factor that wasn’t found in the secretome analysis.

Larvae of the Great wax moth *Galleria mellonella* defenses themselves from bacteria invaders by activating its innate immune system with results in the production of antibacterial peptides. 12 different inducible antibacterial peptides have been identified in the hemolymph of *G. mellonella* so far. It has been shown by Andrejko et al. that Elastase B of *P. aeruginosa* is able to degrade antibacterial peptides in the hemolymph (Andrejko et al., 2009). This would be one explanation why both CTP defective mutants are less virulent during systemic infection because Elastase B activity was significantly reduced in these strains.

In another study of the same group Andrejko showed that Protease IV degrades most likely the apolipoporphin-III (apoLp-III) protein. ApoLp-III is a versatile protein involved in lipid transport and an immune-stimulating protein (Andrejko et al., 2008). ApoLp-III has also hemoagglutinating properties in the hemolymph of *G. mellonella* larvae and plays a role in detoxification of lipopolysaccharide endotoxines (Iimura et al., 1998). The degradation of Apo-Lp-III could also explain the attenuated virulence of the CTP defective strains by systemic infection.

The virulence of the sterile extracellular fraction on the other hand cannot solely explained by these degradation of the antimicrobial peptides or Apo-Lp-III. It has been shown before
that purified protease fraction from \textit{P.aeruginosa} lead to pathogenesis of \textit{G.mellonella} although the precise protease has not been indentified (Kučera and Lysenko, 1968). Our results confirm these observation that toxins secreted by \textit{P.aeruginosa} are lethal for the Greater wax moth and that the CTP defective strains probably secrete less of this toxin.

\section*{Chapter discussion}

The role that the CTP genes \textit{ctpA} and \textit{prc} of \textit{P.aeruginosa} play in pathogenesis of the bacterium was intensively tested in several infection models. Virulence of both \textit{\Delta ctpA} and \textit{\Delta prc} \textit{P.aeruginosa} strains were attenuated in the \textit{A.thalinana} model, the \textit{D.melanogaster} feeding assay and the \textit{C.elegans} slow killing assay. All these models are non-invasive and depend greatly in over winning the hosts own defense mechanisms. There were no clear difference between the two CTP defective strain. These results suggest that the CTP gene products are important in establish full virulence in these model organisms. The wide range genetic origin of the organisms show that the CTPs are involved in a general mechanism that influences pathogenesis of the bacterium. In three other assays the virulence of both the \textit{\Delta ctpA} and \textit{\Delta prc} strains were tested in models with direct systemic infection. For these infections \textit{P.aeruginosa} doesn’t have to overcome the natural physical barrier present in the hosts. This was evaluated in the \textit{D.melanogaster} needle prickling assay and the \textit{G.mellonella} injection assay. In the needle prickling assay with \textit{D.melanogaster}, virulence reduction was not that evident as seen with the feeding assay. There were both difference in survival prolongation for the \textit{\Delta ctpA} and \textit{\Delta prc} strain, which was significant for \textit{\Delta ctpA} but not for \textit{\Delta prc}. Overall killing of the systemic infected \textit{D.melanogaster} is much faster than when fed on \textit{P.aeruginosa} (Median survival time of 29 hours versus 6.1 days). \textit{G.mellonella} was also infected systemically. In this assay both \textit{\Delta prc} and the \textit{\Delta ctpA} strain showed a significant difference in LD\textsubscript{50} values. In this case the \textit{\Delta prc} was less virulent than the \textit{\Delta ctpA} strain. Which is opposed from the observations in the \textit{D.melanogaster} needle prickling assay.

As shown in chapter 5 both CTP inactivated mutants are defective in the secretion of virulence proteases such as Elastase B, Elastase A, Protease VI, Alkaline protease and the Aminopeptidase PepB. Based on the results observed during the infection experiments these secretion defect influences the virulence of \textit{P.aeruginosa}. The virulence protease could
well play a role in the destruction of psychical barriers in the host or other immune responses and by doing so establishing a “porte-d'entrée” for systemic infection. Although other virulence factors can also be involved that haven’t been identified in the secretome analysis yet. But these virulence factors are not solely necessary of entering and establishing the infection as the other systemic infection experiments have shown. There was also a reduced virulence of the two CTP defective strains in these infection assays. Thus the virulence factors play also a role in the systemic infection of organisms. The last experiment even showed a clear role for the secreted virulence factors of \textit{P.aeruginosa} as sterile supernatant cultures were lethal for \textit{G.mellonella}. This confirms that inactivating the CTP of \textit{P.aeruginosa} leads to a decreased virulence by direct influencing the secretion of virulence factors.

Based on these results the CTPs from \textit{Pseudomonas aeruginosa} can be proposed as novel antibiotic targets which results in a secretion defect and virulence reduction.
References


"Details emerge more clearly as the fractal curve is redrawn."

IAN MALCOLM

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff
CHAPTER 7

Results and discussion

CTP target sub cellular localization

This chapter is based on:

Chapter summary

Carboxy-terminal processing proteases are designated as periplasmatic enzymes. This assertion is based on two studies performed on Prc from *E.coli* with poor experiment evidence. The sub cellular localization of CTPs would may give in insight in there physiological function.

Bioinformatic analysis on the deduced protein sequences of CtpA and Prc predict a N-terminal signal peptide that would direct these proteases to be translocated across the inner membrane.

Therefore a sub cellular localization study was performed. A plasmid was constructed that carried the *ctpA* gene under control of a *lac*-promoter that is constitutively transcribed in *P.aeruginosa*.

*P.aeruginosa* strains harboring this plasmid were fractionized and followed by Western blot analysis. The results showed that CtpA is solely localized within the periplasm and not in the extracellular fraction, nor membrane or cytoplasm.

The sub cellular localization of CtpA *P.aeruginosa* in the periplasm gives further insight in the physiological function of this protease.
CTP target sub cellular localization

Knowledge is rather limited about Carboxy-terminal processing proteases in Prokaryotes. The first CTP that was characterized was the Tsp protease ("Tail specific protease") isolated from *Escherichia coli*. The Tsp protease which is now referred to as Prc showed degrading activity towards a λ repressor with non-polar C-termini (Silber et al., 1992). Before the characterization of the inactivated Δprc *E.coli* mutant CTPs were suggested to be involved in the c-terminal cleavage of the Penicillin-binding protein 3 (Hara et al., 1991).

Homologues genes in plants, algae and cyanobacteria have a very distinct and well elucidated function (Satoh and Yamamoto, 2007). In these phototrophic organisms CTPs activate the pre-D1 protein in the thylakoid lumen by C-terminal cleavage. The activated D1 protein plays a crucial role in the energy transfer of Photosystem II.

In bacteria CTPs are involved in several basal physiological functions. There is some doubt about its real sub cellular localization. This information would indeed help in the better understanding of its physiological function and how this influences other cellular processes. In most literature CTPs from Gram-negative bacteria are referred to a periplasmic proteases. When these literature is examined in more detail the experimental evidence of this sub cellular localization is poor. There are two studies that form the fundamentals of this assertion. Both were performed on *E.coli* in the early 1990ies. One study was done by Hara and coworkers in 1991 (Hara et al., 1991). In this paper the localization experiment was carried out with Maxicells. Maxicells are UV irradiated *E.coli* strains which therefore have damaged and nonfunctional genomic DNA. These Maxicells cells can transformed and the gene product will be synthesized via an *in vitro-coupled* transcription-translation system in the presence of $^{35}$S-methionine. Therefore only gene products of the plasmid are radioactive. In this localization experiment the prc gene was cloned on a plasmid, transformed and the gene product was synthesized. The subsequent sub cellular fractionation showed that Prc was localized predominantly in the periplasm but also in the cytoplasmic fraction and the cytoplasmic membrane. The authors suggested that a detailed follow-up sub cellular localization experiment would be necessary.

The second study was done by Silber and coworkers (Silber et al., 1992). In these experiments Prc was overexpressed in an *E.coli* strain. Periplasmic and cytoplasmic/membrane fractions
were separated by osmotic shock. After fractionation, overexpressed Prc could be shown in the periplasmic and cytoplasmic/membrane fraction.

Both experiments were carried out under rather non physiological conditions that could influences the outcome. Besides that both groups didn’t take in account that the Prc could be secreted in the extracellular environment. This hypothesis would be interesting based on the results presented in chapter 5 and 6. In chapter 5 was shown that the CTP defective mutans of P.aeruginosa were defective in the secretion of several virulence factors. Further in Chapter 6 was shown that the CTP of P.aeruginosa played a role in the virulence of the bacterium. What the exact mechanism is which could explain these results remains unknown. In a study about the Gram-negative pathogen Chlamydia trachomatis it was shown that the CTP was involved in the interfering of the NF-κB pathway in the human host inflammatory response by processing of the intracellular p65 protein (Lad et al., 2007). C.trachomatis is an obligate intracellular human pathogen. This interference with the NF-κB pathway could only be physiological possible if the CTP would be secreted by the organism.

Therefore it was interesting to localize CtpA of P.aeruginosa.

**Bioinformatic secretion analysis**

Most secreted proteins from Gram-negative bacteria have a N-terminal signal peptide that directs these proteins to be translocated across the cytoplasmic membrane. These N-terminal signal peptides can be predicted by bioinformatic tools.

In figures 7.1 and 7.2 are the results shown of the N-signal peptide prediction by the SignalP software for CtpA and Prc of P.aeruginosa (Petersen et al., 2011).
Figure 7.1: Plot of the SignalP N-terminal signal prediction of Prc.

Table 7.1: Output parameters of SignalP analysis Prc

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</table>

Cleavage site between pos. 23 and 24: LFA-ST D=0.884 D-cutoff=0.570
Chapter 7

Figure 7.1 shows the SignalP plot for the N-signal prediction for Prc. In table 7.1 are the output parameters defined. The bioinformatic results show that Prc has a predicted N-terminal signal of 23 amino acids. Figure 7.2 shows the SignalP prediction results for CtpA and the output parameters are stated in table 7.2. Also CtpA has a predicted N-signal peptide of 23 amino acids. The results support the hypothesis that the proteases are translocated across the cytoplasmic membrane.

Sub cellular localization of CtpA

Experiments were carried out in order to determine the sub cellular localization of CtpA. Therefore peptide specific polyclonal antibodies were designed. The antibodies were designed based on two 16 amino acids long peptides. These epitopes were synthesized and conjugated to keyhole limpet haemocyanin carrier proteins and antibodies were raised in rabbits. The presence of CtpA was investigated by Western blot analysis.

*P. aeruginosa* wild type strain was grown and but no CtpA could be detected in whole cells or extra cellular fractions by Western blotting. Also variation of culture conditions such as different growth time, culture medium or temperature did not result in detection of CtpA. Speculating about these findings, this could be the result of very low amount of CtpA in the cell, or that the CtpA is only present at a certain time frame within the cell cycle.

To overcome this problem a plasmid was constructed with the *ctpA* gene, the pCtpA-lac. The *ctpA* gene was not cloned having its own promoter but was under the control of a lact promoter. The lac-promotor in *P. aeruginosa* is constitutively transcribed which results in a moderate expression (Rosenau and Jaeger, 2004).

The pCtpA-lac was transformed to *P. aeruginosa* by biparental filter matings. The *P. aeruginosa* strain harboring the vector was grown for 4 hours and was subsequent fractionated and a Western blot analysis was performed. The results of the sub cellular localization is shown in figure 7.3.
Figure 7.2: Plot of the SignalP N-terminal signal prediction of CtpA.

Table 7.2: Output parameters of SignalP analysis CtpA

<table>
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Cleavage site between pos. 23 and 24: AQA-AD D=0.914 D-cutoff=0.570
In lane 3 (PP) of figure 7.3 it is shown clearly that the CtpA of *P. aeruginosa* could be detected in the periplasmic fraction. The disulfide oxidoreductase DsbA was also blotted as a control for the periplasmic fraction which could also be detected (Lory et al., 1983). CtpA was not detected in the cytoplasmic or the cytoplasmic membrane (see fig. 7.3: lanes 4 and 5). The groups of Hara and Silber both found Prc of *E. coli* also in the cytoplasmic and cytoplasmic membrane (Hara et al., 1991; Silber et al., 1992). This is probably caused by these relatively unnatural conditions and the massive over expression in the used bacterial strains. In this experiment the *ctpA* gene was under control of the lac-promotor which gives a very moderate expression. Lane 5 shows the extracellular fraction in which CtpA could not be detected. Endotoxine A antibodies were used as a control for the extracellular fraction which detected Endotoxine A (Urban et al., 2001). The positive controls confirm that the fractionations were correctly executed.
Figure 7.3: Subcellular localization of CtpA a CTP-3 from *Pseudomonas aeruginosa*. Whole cell (WC; lane 1), extracellular (EC; lane 2) and the cell fractions: periplasm (PP; lane 3), inner and outer membrane (M; lane 4) and cytoplasm (CP; lane 5) were blotted with protein-specific antisera. Antisera of disulphide oxidoreductase A (DsbA) and exotoxin A (ETA) were used as controls for the PP and EC fractions, respectively.
Chapter discussion

CtpA of *P. aeruginosa* is sub cellular localized in the periplasm. This observation rejects the hypothesis that CtpA could be secreted in the extracellular environment. The hypothesis was regenerated on the results of Lad and coworkers which showed that Tsp from *C. trachomatis* interfered with the NF-κB pathway by cleaving the p65 protein of the host in a human cell line. This could explain how these obligate intracellular pathogens could evade the host own immune system. The most simple explanation would be that the Tsp of *C. trachomatis* facilitates this under the conditions that the protease is secreted in the extracellular environment or would be localized at least in the outer membrane of the pathogen.

This clearly not the case for *P. aeruginosa* as the CtpA is solely localized in the periplasm. This gives additional information in the physiological function of CTPs in *P. aeruginosa*. As mentioned in Chapter 5 CTPs could be an effector proteases that are secreted into the extracellular environment to regulate virulence factors. For example could the CTP proteases be secreted outside and cleave virulence factors such as Elastase A, B or protease IV and Alkaline protease and by doing so inactivate them. Because CtpA it is now shown to be localized in the periplasm this hypothesis is rejected. No direct effect of the protease can be expected in the extracellular environment, this effect has to be related to a periplasmic action of the protease.

The other CTP from *P. aeruginosa* Prc has not been sub cellular localized. Although it is plausible to assume that Prc is localized in the periplasm.

Several physiological functions have been suggested for bacterial CTPs. One function is the involvement of CTPs in the tmRNA protein-tagging system. This bacterial system controls the degradation of incorrectly synthesized proteins. Keiler and coworker suggested that Prc of *E. coli* is involved in this system (Keiler et al., 1996). In this system, an tmRNA tag is added to mRNAs when ribosomes are stalled due to a lack of termination codons. The resulting C-terminal tmRNA signal peptide tagged periplasmic protein is then recognized by Prc and subsequently degraded. This system is solely functional in the periplasm which would support the observation that CtpA is localized in the periplasm. Prc of *E. coli* has also been shown to degrade the lipoprotein NlpI which is anchored in the outer membrane of *E. coli* facing the periplasm. This support the localization results.
It is also suggested that Prc from *E.coli* cleaves Penicillin-Binding-Protein 3 (PBP3). PBPs are periplasmatic enzymes involved in the biosynthesis of the cell wall. *E.coli* PBP-3 is thought to be a key element in cell septation in which it presumably initiates polymerization of the septum peptidoglycan by catalyzing a transpeptidation reaction during cell division (Nguyen-Distèche et al., 1998). Based on this a new hypothesis can be introduced that CtpA and Prc of *P.aeruginosa* are involved in the processing of PBP3. This would be in evolutionary and functional equivalence to CTPs from phototrophic organisms. CTPs from Gram-negative bacteria may be required to activate periplasmatic proteins by cleavage as the photosynthetic D1 protein is activated in plant cells. The localization of CtpA in the periplasm of *P.aeruginosa* supports the hypothesis. One can hypothesize that the formation of the septum is a crucial step during cell cyclus which would needed to be highly regulated and controlled. The CTPs could act as an activator of PBP3 to ensure an instant and onsite activation of the process. *De novo* synthesis of PBP-3 in a directly active form and subsequent translocation to the periplasm would probably be to slow or may lead to undesired side reactions within the cell. This hypothesis will be further elucidated in Chapter 9.
References


CTP target sub cellular localization
"Inevitably, underlying instabilities begin to appear."

IAN MALCOLM* 

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luisingh-Sijthoff
CHAPTER 8
Results and discussion

CTP target isolation: cloning, expression and activity of CtpA and Prc

This chapter is partly based on:

Chapter summary

CtpA and Prc were cloned and expression in *E.coli* production strains was initial unsuccessful under the experimented conditions. CtpA was ones successfully expressed in a *E.coli* JM109(DE3) strain but DNA sequencing revealed a mutation within the approximation of the active site which probably made it inactive. CtpA and Prc were successfully expressed and purified by a *in vitro* transcription-translation system. Afterwards expression condition in *E.coli* BL21(DE3) were eventually optimized which led to successful expression of both CTPs in this host.

CtpA and Prc both showed proteolytic activity towards the β-casein protein (25 kDa) resulting in two distinct cleavage products of 20 and 15 kDa.

Alanine substitution mutagenesis experiments revealed that two predicted active residues serine and lysine within the catalytic domain of the proteases are important for the proteolytic activity. CtpA-S302A, CtpA-K327A, Prc-S479A and Prc-K504A variants didn't show any activity towards cleavage of β-casein.
**Cloning, expression and activity of CtpA and Prc**

CtpA and Prc are putative proteases from *Pseudomonas aeruginosa*. The first CTP that has been cloned was Prc from *Escherichia coli* (Silber et al., 1992). Silber and coworkers cloned Prc, over expressed and purified the protease. Protease activity was shown by degradation towards the #105 protein which is a variant of the λ-repressor protein. #105 has a nonpolar C-terminal (amino acid sequence: WVAAA) in compare to the wild type λ-repressor protein (amino acid sequence: RSEYE). Typical protease inhibitors such as 3,4-DCI, 3,4-dichloroisocoumarin (3,4-DCI), phenylmethylsulfonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone(TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK ), o-phenanthroline (o-PA) did not affect the protease activity.

As shown in Chapter 6 the CTPs from *P. aeruginosa* can be regarded as novel antibiotic targets. For the elucidation of the physiological function of the protease and for the development of possible inhibitors it necessary to clone and characterize these proteases. Therefore CtpA and Prc were cloned and expressed.

**Over expression and purification of CtpA and Prc**

CtpA and Prc were cloned in a pET19b vector. The pET19b is a vector with a N-terminal His-tag followed by an enterokinase site and three cloning sites, T7 expression system, pBR322 origin and bla antibiotic resistance gene (Novagen, Darmstadt, Germany). A 1,265 bp PCR fragment was amplified from the ctpA open reading frame (ORF5134) starting at the ctpA start codon (ATG) and ended with the stop codon (TGA) flanking both sides with NdeI (‘5) and XhoI (‘3) restriction sites. The PCR fragment was cloned into the pET19b via the NdeI/XhoI restriction sites. Prc was cloned in a similar way. A 2,051 bp PCR fragment was amplified from ORF3257 starting at the prc start codon (ATG) and ended with the stop codon (TGA). The PCR fragment was cloned into the pET19b via the NdeI/XhoI restriction sites.

The plasmids were transformed to chemical competent *E.coli* DH5α cells for plasmid production and isolation. The plasmids were transformed to chemical competent *E.coli* BL21(DE3) cells for over expression. Production strains were always grown in LB medium with the presence of 4% glucose in order to suppress the lac-promoter.
The transformation of the over expression plasmid harboring ctpA and prc were not successful in the beginning. Only a few or no transformed colonies were retained after growing the transformation plates at 37 °C. After picking, clones were grown overnight in LB medium and an over expression culture was prepared from a overnight culture at starting OD of 0.05 and grown in the presence of 4% glucose to an OD of 1.0. The over expression was started by adding 0.5 mM IPTG. No over expression could be observed.

In order to accomplish over expression, different growth condition and strains were tried. Transformation of pET19b-ctpA in E.coli JM109(DE3) resulted in a clone that was able to over express CtpA using auto induction medium grown for 16 hours at 37 °C figure 8.1. The clone of the over expression strain E.coli JM109(DE3) with pET19b-CtpA was sequenced. The results of the sequencing is shown in figure 8.2.

In figure 8.1 is shown that the plasmid pET19b-CtpA that was transformed to E.coli JM109(DE3) was successful in the over expression of CtpA. But the sequenced plasmid for the over expression strain had a mutation at amino acid position 300 (see fig. 8.2). Serine 300 was mutatated to arginine 300 (S300R). The mutation lies within the predicted proteolytic active site of the enzyme. The active residues are predicted as serine 302 and lysine 315. The distance between the active site residue serine 302 is 2 amino acids. Serine is a nucleophilic amino acid with a small side chain with a hydroxyl group. Asparagine is a basic amino acid with a long side chain. It is therefore plausible that the mutation resulted in an inactive variant of CtpA.

Because of the difficulties to over express both CTPs and the gained mutation within the over expressed CtpA hinds that these proteases negatively influences the overproduction E.coli strain.

Based on these results the over expression was carried out using a in vitro transcription-translation system. Both CtpA and Prc were expressed using the system. The synthesized proteins were subsequently purified over the hexahistidine tag using nickel nitrilotriacetic acid affinity resin magnetic beads. The results of the CtpA over expression and purification are shown in figure 8.3.
Figure 8.1: SDS-PAGE gel: over expression of CtpA in *E. coli* JM109(DE3) in auto induction medium grown for 16 hours at 37 °C. The arrow indicates the overexpressed protein with a size of approximately 45 kDa.

Figure 8.2: DNA sequence of clone pET-CtpA-S300R. A: DNA sequence; above ctpA, below clone S300R. B: translated amino acid sequence; above CtpA, below CtpA-S300R. Mutation S300R is indicated with the arrow. The putative active residues of the proteolytic active site are marked with asterisks.
As shown in lane 6 (fig. 8.3A; E) CtpA could be expressed with the in vitro transcription-translation system and purified over the Ni-NTA affinity resin. In the crude extract in lane 2 the synthesized CtpA with a protein band of about 45 kDa can been seen. In the wash fractions (lanes 4 and 5) no CtpA can be seen. The final protease solution was nearly pure. The purified 6his-CtpA could also be detected by Western blotting with the polyclonal rabbit antiserum (see fig. 8.3B).

Prc was expressed in a similar way as CtpA using in vitro transcription-translation and subsequent purified with the Ni-NTA affinity resin.

To gain higher amounts of protease, over expression in a bacterial strain is preferable. Therefore further experiments were executed in order to optimize over expression of CtpA and Prc in a E.coli production strain. These optimizations lead to a over expression protocol with distinct conditions. Plasmids pET19b-CtpA and pET19b-Prc were transformed to chemical competent E.coli BL21(DE3) cells and grown in plates with 4% glucose at room temperature. Colonies harboring the over expression plasmids were grown overnight in LB medium with 4% glucose at 24 °C. An over expression culture was inoculated at OD580 0.05 and grown for 4 hours in LB medium with 4% glucose. Expression was induced with 1 mM IPTG. After 1 hour cells were harvested for purification.

The results of the over expression of CtpA and Prc in E.coli BL21(DE3) are shown in figure 8.4. In lane 2 the over expression of Prc can be seen in the crude extract with a high density protein band between 70 and 100 kDa. The lane 8 the over expression of CtpA is visible as a high density protein band of about 45 kDa. The subsequent lanes show the purification of both proteases over the hexahistidine tag using nickel nitrilotriacetic acid affinity column resin. In the flow trough fraction of the Prc over expression a high amount of the overexpressed Prc protease can be seen. This is probably due to overloading of the affinity resin capacity. In the following washing steps only a small amount of Prc was lost. The elution fractions show a almost pure Prc protease with a small amount of impurifications between the 40 and 55 kDa. The purification of CtpA shows a similar result. Although no obvious amounts of lost CtpA in the flow through fraction could be observed. The elution fractions show a almost pure CtpA.
CTP target isolation: Cloning, expression and activity of CtpA and Prc

Figure 8.3: A. CtpA expression with an in vitro transcription-translation system and Ni-NTA purification. M: protein marker. CE: crude extract. FT: flow trough. W1: wash step 1, 20 mM imidazole. W2: Wash step 2, 20 mM imidazole. E: elution, 250 mM imidazole. 6His-CtpA is indicated with the arrow. B. Western blot of 6his-CtpA with polyclonal CtpA rabbit antiserum.

Figure 8.4: Expression of Prc and CtpA in E.coli BL21 (DE3) at 24 °C and subsequent Ni-NTA purification. M: protein marker. CE: crude extract. FT: flow trough. W1: wash step 1, 20 mM imidazole. W2: Wash step 2, 20 mM imidazole. E1: elution, 250 mM imidazole. E2 10x concentrated elution fraction.
Protease activity of CtpA and Prc

CtpA and Prc are putative C-terminal processing proteases but these CTPs from *P. aeruginosa* had never been expressed and purified before, let alone shown activity. In order to detect any activity from the CtpA and Prc general protease assays were performed. Also it was unknown if CtpA and Prc were actively expressed and purified.

**Figure 8.5** shows a general casein assay with CptA and Prc. The composition of the assay except the substrate was performed according to the #105 assay from Silber and coworkers (Silber et al., 1992). The assay buffer consisted of 4 μM β-Casein solution in 20 mM Tris (pH 8.0) and 200 mM potassium chloride. 1 μg of each protease CtpA and Prc was added were incubated for 16 hr at 37°C. The assay was executed in triplicates.

In this assay no proteolytic activity could be observed from CtpA or Prc. If the β-casein was degraded a more or less clear solution would have developed. The assay with trypsin was incorporated as a positive control. In lane 2 can been seen that trypsine cleavages the β-casein and the reaction results in a clear solution.

So these result didn’t showed any activity from the CtpA and Prc. Information of CTPs from plants such as CtpA from Spinach or CtpA form the algae *Scenedesmus obliquus* elucidated that these protease only cleave a distinct peptide from the carboxy-terminal of the D1 protein. If CTPs from bacteria such as *P. aeruginosa* would have a similar function it could be so that substrates are only cleaved at specific sites. Therefore cleavage could not be observed in the β-casein assay describes above.

So the β-casein assay was executed again and the reaction mixture was analyzed on a SDS-PAGE gel. The results of this β-casein assay are shown in **figure 8.6**. In lane 2 the substrate β-casein can be seen. β-Casein is a protein from bovine milk with a molecular weight of 25 kDa which corresponds with the protein marker. In lane 3 and 4 the cleavage of β-casein can been observed by CtpA and Prc. The β-casein substrate band has disappeared and two distinct protein bands can be observed with smaller molecular weight of about 20 en 15 kDa. These protein bands are degradation products of the β-casein.
FTTP target isolation: Cloning, expression and activity of CtpA and Prf

Figure 8.5: β-casein assay. Proteases CtpA, Prf and Trypsin (Trp) were incubated for 16 hours at 37°C. The assay consisted of 1 μg protease, 4 μM β-casein, 20 mM Tris and 200 mM KCl (pH 8.0). Lane 1: solely β-casein; lane 2: β-casein with trypsin; lane 3: β-casein with CtpA; lane 4: β-casein with Prf.

Figure 8.6: β-casein assay with CtpA and Prf. The assay consisted of 1 μg protease, 4 μM β-casein, 20 mM Tris and 200 mM KCl (pH 8.0). Lane 1: protein marker (M); lane 2: solely β-casein; lane 3: β-casein with CtpA; lane 4: β-casein with Prf.
The degradation products probably do not represent the N-terminal and C-terminal of the cleaved β-casein because the two band together make a protein of about 35 kDa which is much bigger than the β-casein substrate of about 25 kDa. The most evident explanation would be that CtpA and Prc cleavage the β-casein in a protein of about 20 kDa and is subsequently cleaved again in protein of 15 kDa. The other smaller protein degradation products of about 5 kDa can’t be seen on the SDS-PAGE gel. Another distinct result is that both proteases seem to cleave the β-casein in the precise the same products. Although this can only be concluded from the observation of the same molecular weights of the protein bands. This assay shows that the expressed and purified proteases CtpA and Prc are active. This assay proofs only that the proteases are functional but β-casein is clearly not the physiological substrate for these proteases as it is from bovine origin.

**Evaluation of active residues of CtpA and Prc**

Based on multiple alignment analysis and the elucidation of the crystal structure of CtpA from *Scenedesmus obliquus* it is suggested that CTPs have two active residues within the active proteolytic site (Liao et al., 2000). Figure 8.7 shows the multiple alignment of the active site of some pathogen Gram-positive and Gram-negative bacteria.

The alignment shows that a serine and lysine (see fig. 8.7: indicated with the red boxes) are highly conserved in CTPs from bacteria. Based on the knowledge of CptA from plants and algae the catalytic mechanism is proposed to consists of a Serine/ Lysine dyad. No mutagenesis studies have been performed on bacterial CTPs. Therefore the putative active residues CtpA and Prc were subjected to alanine substitution mutagenesis. The codon of serine 302 in plasmid pET19b-CtpA was altered into an alanine with site-directed mutagenesis forming pET19b-CtpA-S300A and lysine 327 to alanine forming pET19b-CtpA-K327A. The codon of serine 479 in plasmid pET19b-Prc was altered into an alanine with site-directed mutagenesis forming pET19b-Prc-S479A and lysine 504 to alanine forming pET19b-Prc-K504A. The plasmids were transformed, multiplied and isolated from a *E.coli* DH5α strain. The plasmids were subsequently used over expression of the mutant protease with a *in vitro* transcription-translation system.
CTP target isolation: Cloning, expression and activity of CtpA and Prc

Figure 8.7: Multiple alignment of selected amino acid sequences within the active proteolytic site of CTPs. The conserved active residues serine and lysine are marked with the red boxes and the asterisks. S41 subfamilies are designated CTP1, CTP3 and CTP7. Prc Pseudomonas aeruginosa (Pa; PA3257); Prc Escherichia coli (Ec; M75634.1); Prc Chlamydia trachomatis (Ct; YP_007735755); CtpA Pseudomonas aeruginosa (Pa; PA5134); CtpA Brucella suis (Bs; NP_698817.1); CtpA Legionella pneumophila (Lp; YP_122899.1); CtpB Enterococcus faecalis (Ef; NP_815387.1) and CtpB Listeria monocytogenes (Lm; YP_007607420.1).

Synthesized proteases were purified with Ni-NTA affinity resin on magnetic beads. The mutant CtpA and Prc were subjected to a β-casein assay. The results of the β-casein assay are depicted in figure 8.8. In lane 3 (see fig. 8.8) the cleavage of β-casein by CtpA can be seen in the two districts cleavage products of 20 and 15 kDa. In lane 4 and 5 the cleavage assay of the two alanine substitution mutants of CtpA are depicted. Both CtpA-S302A and CtpA-K327A were not able to cleave β-casein. The mutation of the serine and lysine to alanine resulted in inactive variants of CtpA.

The cleavage of β-casein by Prc is shown in lane 7 giving two cleavage products of 20 and 15 kDa. Also the two alanine substitution mutants Prc-S479A and Prc-K504A were not able to cleave the β-casein. This is comparable of as seen with the CtpA substitution mutants.

Both serines in CtpA and Prc on this specific location within the active site is important for proteolytic activity. The same is valid for the lysine within this active site. If one of the two amino acids is exchanged for an alanine no cleavage activity towards β-casein can be seen. Although β-casein is not the physiological substrate of P. aeruginosa for these proteases this experiments will be representative for its cleavage mechanism. There is a possibility that the proteases were not active because of faults during the in vitro transcription-translation system. Therefore the plasmid was sequenced in order to check if there were unwanted mutation and the synthesis was executed in combination with the CtpA and Prc and treated equal. Both CtpA and Prc were active as shown in figure 8.8 and they act as a positive control of the synthesis. The observed inactivity of the proteases can therefore be attributed to the alanine substitutions.

Figure 8.9 shows the elucidated crystal structure of CtpA from Scenedesmus obliquus (Liao et al., 2000). Here the two active residues of serine 372 and lysine 397 can be seen in the active site (depicted in blue). The PDZ domain is colored yellow and the N-terminal structure pink. The active dyad mechanism functions in the way that lysine 397 acts as a base catalyst to the serine 372 nucleophile. These result of CtpA and Prc from P. aeruginosa suggested that a similar proteolytic mechanism is applicable.
Figure 8.9: Structure of the CtpA from *Scenedesmus obliquus*. Catalytic domain is in blue, the PDZ domain in yellow and the N-terminal domain in pink. The active residues serine S372 and lysine K397 are shown as ball-and-prick model representations (Liao et al., 2000).
Chapter discussion

The CTPs from *P. aeruginosa* were successfully cloned, expressed and purified. As an identified novel antibiotic target these proteases need to be characterized in order to explore their physiological function and for the development of inhibitors. The cloning and expression was difficult in several *E. coli* strains. It seems that the over produced production strains experienced difficulties under certain expression conditions probably because the proteases interfered with physiological functions of the bacterium. This was illustrated by the fact that the only *E. coli* clone that overproduced CtpA contained a mutation within the approximation of the active residues. Which probably resulted in an inactive variant. These results confirm the hypothesis that CTPs are involved in basal cellular functions. Especially Prc from *P. aeruginosa* has a high resemblances to Prc from *E. coli* (38% identity and 50% similarity) which could be physiological active in this host.

Therefore non-viable expression system were tried such as *in vitro* transcription and translation system in order to synthesis the desired proteases. Using these systems it was possible to synthesis the proteases CtpA and Prc and subsequent purification. Afterwards both proteases were successfully expressed in the *E. coli* BL21(DE3) under much milder conditions such as lower temperature and strict control of promoter leakage.

There was no evidence that the synthesized proteases were active. Activity of both CptA and Prc could be proven with a β-casein assay. CtpA and Prc both cleaved β-casein forming to products of 20 and 15 kDa. The two products are probably subsequent cleavage products. This assay proofs that the expressed and purified proteases were active.

Further characterization was performed by mutagenesis of the proteases CptA and Prc. Putative active residues as predicted by multi-alignments analysis and indentified conserved amino acids within the catalytic domain. Alanine substitution mutation of the active residues serine 302 and lysine 327 in CtpA and serine 497 and lysine 504 were constructed, expressed and purified. These substituted variants of CtpA and Prc showed no activity in the β-casein assay. This indicates that both predicted active residues are indeed important for proteolytic activity in the cleavage of β-casein. The proposed mechanism that involves the serine and lysine forming a catalytic dyad maybe well valid for until now unknown physiological substrates.
References


"Flaws in the system will now become severe."

IAN MALCOLM

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff*
CHAPTER 9
Results and discussion

CTP target characterization: physiological function of CtpA and Prc in Pseudomonas aeruginosa

This chapter is partly based on:

Chapter summary

Physiological substrates of bacterial CTPs are unknown though some substrates have been suggested. One substrate is the Penicillin Binding Protein 3 (PBP3) which has been investigated as a substrate for Prc in *E. coli*. A C-terminal cleavage site was determined by research done with *prc* negative strains. *P. aeruginosa* has two distinct PBP3 proteins, named PBP3 and PBP3A.

Based on chemical synthesized peptide substrates CtpA was able to cleave both PBP3 and PBP3A. Prc on the other hand was only capable in processing PBP3A. Specificity experiments showed that CtpA had a higher relative activity towards PBP3 than for PBP3A. This suggests that Prc can process primarily PBP3A and CtpA prefers PBP3 indentifying these proteins as possible physiological substrates.

Prc from *E. coli* was shown to be capable in processing the PBP3 from *E. coli* and confirming the proposed cleavage site of the protease in previous observations.

Additional experiments showed that both CtpA and Prc could additional be involved in the tmRNA tagging system. This was based on cleavage experiments of these CTP proteases with a tmRNA synthetic peptide substrate.

The results of this chapter were obtained in collaboration with:

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Physiological function of CtpA and Prc in *Pseudomonas aeruginosa*

In Chapter 5 it was shown that inactivated ΔctpA and Δprc mutants of *P.aeruginosa* are attenuated in the secretion of proteins especially in virulence factors as analysed by 2D gels electrophoresis. The secretome analysis was confirmed by enzyme specific assays of the virulence factors Elastase B, Elastase A, Protease VI, Alkaline protease and Aminopeptidase PepB. In Chapter 6 it was shown that the CTP defective mutants were also attenuated in its pathogenesis as shown in different infection models including *A.thaliana*, *D.melanogaster*, *C.elegans* and *G.mellonella*. In order to explain these effects it is necessary to elucidate the cellular function of the CTP proteases CtpA and Prc. In Chapter 7 it was shown that CtpA of *P.aeruginosa* is sub cellular localized in the periplasm of *P.aeruginosa* and not in the cytoplasm nor extracellular environment. This suggests that a cellular function has to be located within the periplasm of *P.aeruginosa*. Although no experiments were performed on the localization of Prc is it presumed that this protease is also localization within the periplasm. In Chapter 8 both proteases have been functional expressed and purified. Besides that is was shown that CtpA and Prc have proteolytic activity confirming the enzymes as proteases. This gives further information that a physiological function has to be involving proteolytic activity within the periplasm of *P.aeruginosa*.

Other bacterial CTP mutants showed a broad range of pleiotropic phenotypes. In *Escherichia coli* a non-functional prc gene resulted in a strain that is not able to grown at low temperatures and under osmotic stress. Low or even medium osmolarity of the medium causes the bacteria to lyse. The inactive prc mutant also showed leaking of periplasmic proteins (Hara et al., 1991). Increased antibiotic susceptibility has also been shown as a result of functional loss of prc (Seoane et al., 1992). Inactivation of ctpA in *Rhizobium leguminosarum* led to a decreased desiccation tolerance (Gilbert et al., 2007).

Silber and co-workers showed that Prc from *E.coli* degrades the #105 protein a variant of the λ-suppressor protein (Silber, 1992). The #105 protein has a more hydrophobic carboxy-terminal (amino acids sequence: WVAAA) than the λ-suppresor (amino acids sequence: RSEYE). Whether Prc cleaves the #105 also in vivo is unclear. The #105 variant is at least being processed in vivo in inactivated prc mutants (Keiler et al., 1995). Prc of *E.coli* is involved in the
processing of TonB and the Arc repressor. TonB is a periplasmic protein that is involved in the binding of outer membrane proteins (Keiler et al., 1995).

Tadokoro and co-workers showed that Prc of *E.coli* interacts with the lipoprotein NlpI which seems only be functional when the C-terminal is processed. NlpI may be involved in septum formation or in cell wall degradation during cell division (Ohara et al., 1999). Keiler and co-workers proposed that Prc of *E.coli* is involved in the tmRNA-tagging system. In this system ribosomes that are stalled during synthesis of a protein because of a lack of a termination codon are tagged with a tmRNA peptide. Periplasmic proteins that are tagged and translocated to the periplasm are recognized by Prc and degraded (Keiler et al., 1996).

Most evidence for a physiological substrates of CTPs are the Penicillin-binding-protein 3 (PBP3) from *E.coli*. The PBP3 belongs to the large family of penicillin-binding proteins which play an important role in cell wall synthesis (de León et al., 2010). Penicillin-binding proteins are classified in high molecular weight (HMW) or low molecular weight (LMW) proteins. HMW-PBPs posses an N-terminal domain which is anchored in the cytoplasmic membrane within the periplasm and a C-terminal part which comprises a penicillin-binding domain. The PBP3 plays a role in cell septation and has three distinct functional groups. The first is the N-terminal anchor by which the protein is embedded in the cytoplasmic membrane. The anchor is translocated over the cytoplasmic membrane and comprises also a cytoplasmic moiety. The anchor is essential for full functionality which is suggested to have signal transduction properties. The second domain is the Penicillin binding (PB) domain, which is able to bind penicillin, and is involved in the peptide cross-linking of muramoyl peptides during formation of the peptidoglycan sacculus of an intact cell wall in Gram-negative bacteria (de León et al., 2010). The third domain lies in between the C-terminal PB domain and the N-terminal anchor and is named the non-Penicillin binding domain (nPb). Also the nPB domain is essential for functioning of the PBP3 and is probably acting as a chaperone for the PB domain which can’t fold properly by itself and the nPB is involved in the formation of the PBP3 dimer (Nguyen-Distèche et al., 1998). The PBP3 forms part of the divisome and encompasses the cytoplasm, the inner membrane and the periplasm (see fig. 9.1). PBP3 is not involved in the initiation of division, but in its continuation and completion (Nguyen-Distèche et al., 1998).
Figure 9.1: A. Schematic representation of the divisome. The Fts Z ring forms within the cytoplasmic and when the cell constricts, the diameter of the FtsZ ring decreases as it maintains its position at the leading edge of the septum. The divisome subassemblies encompasses the cytoplasm, the inner membrane and the periplasm. B. Schematic representation of the proteins that form the divisome including PBP3 (Nguyen-Distèche et al., 1998).
PBP3 has been shown to be presented throughout the cell cycle but experiments with cephalaxin have shown that function is only disrupted during cell division. This assumes that PBP3 is specifically activated during the division process (Nguyen-Distèche et al., 1998).

Nagasawa and co-workers proposed that Prc of *E. coli* cleavages 11 amino acids from the C-terminal of the *ftsI* gene product PBP3. This was done by the identification of a precursor PBP3 protein in a prc mutant *E. coli* strain by peptide mapping (Nagasawa et al., 1989). It is unknown if there is a direct interaction between Prc and PBP3 as no direct cleavage assays were performed before.

I therefore hypothesize that CTPs cleave PBP3 proteins and by doing so activating the protein based on the augments mentioned above. This is in analogy to the function of CtpA in plants and Algae that activates the D1 protein of the Photosystem II.
Figure 9.2: Multiple alignment of PBP3: PBP3 and PBP3A of *Pseudomonas aeruginosa* (Pa) and PBP3 from *Escherichia coli* (Ec). Deep purple colour represent identical amino acids, lilac colour represents similar amino acids. The orange bar depicts the non-PB domain and the pink bar the PB domain.
Cleavage of PBP3 by CTPs

Genome mining of *P. aeruginosa* revealed two possible penicillin binding protein genes similar to PBP3 from *E.coli*. The first is named Penicillin Binding Protein 3 with an identity of 45% to PBP3 of *E.coli* and the second is Penicillin Binding Protein 3A with an identity of 42% (see fig. 9.2). The finding of two possible PBP3s matched the presence of two CTPs in *P. aeruginosa* which made the hypothesis stronger.

To test if the CTPs were able to process PBP3 and PBP3A, synthetic peptides were designed with a fluorescent label which enables detection. The peptides represented the C-terminal part of the PBP3 and PBP3A protein with length of 38 amino acids and 29 amino acids respectively. The chemical structure and amino acids sequences are depicted in figure 9.3. Previous observations were based on experiments with Prc of *E.coli*. Therefore the Prc from *E.coli* was also investigated within these experiments.

The PBP3 peptides substrates were incubated with the proteases CtpA and Prc and Prc of *E.coli* at 37°C. The results of these cleavage experiments are shown in figures 9.4-5. Because of the fluorescent labelling and detection at a specific wavelength only peptides which are labelled can be seen on the chromatogram.

In figure 9.4 the cleavage of PBP3 can been seen. The unprocessed substrate has a retention time of 20.2 min (see fig. 9.4A). Cleavage of CtpA results in an peak with a shorter retention time of 19.2 min (see fig. 9.4B). Prc was unable to cleave the PBP3 protein (see fig. 9.4C). No cleavage product could be observed. At the cleavage of Prc from *E.coli* a very small cleavage product could be seen at 19.8 min (see fig. 9.4D).
Figure 9.3: FITC labelled peptide substrates representing the C-terminus of Penicillin Binding Proteins PBP3 and PBP3A from *P. aeruginosa* and PBP3 of *E. coli*. 
Figure 9.5 shows the cleavage of the PBP3A peptide. At the cleavage of CtpA one distinct cleavage peak can be seen with a shorter retention time of 19.2 min. than the substrate peak at 19.5 min (see fig. 9.5A and B). Prc was also able to cleave the PBP3A peptide (see fig. 9.5C). In this chromatogram two cleavage products can be seen. One cleavage peak has the same retention time as the CtpA cleavage product with 19.2 min. The other cleavage peak has a slightly shorter retention time of 19.0 min. No unprocessed cleavage peak could be observed at 19.5 min (see fig. 9.5C). Prc from E. coli also showed activity toward the PBP3A peptide of P. aeruginosa (see fig. 9.5D). In the chromatogram one cleavage product can be seen with again the same retention time of 19.2 min. as the cleavage products of CtpA and Prc.

The cleavage results were further examined by identifying the cleavage sites of the peptides by the use of liquid chromatography coupled to mass spectrometry. The cleavage sites of the two peptides are elucidated and depicted in figures 9.6-9. Only CtpA showed distinct cleavage of PBP3 and was therefore subsequent investigated with LC-MS. The result can be seen in figure 9.6. The chromatogram shows several peaks. The unprocessed substrate peak could not be identified anymore because of the complete cleavage in this assay. The peak at 19.2 had a mass of 723 (see fig. 9.6B) which corresponds to the hydrolysed FTIC-Acp-MA portion. This cleavage product corresponds to the cleavage product seen with the HPLC analysis because it contains the FITC group. The corresponding counter cleavage product was found in the peak at 12.4 min. which has a mass of 3272 (GALRLMNVPDNPLTATEQQQVNAAPAKGGRG) (see fig. 9.6C). Figures 9.7-9 shows the LC-MS cleavage results of the PBP3A peptide. As known from the HPLC results the PBP3A peptidase was cleaved by all three proteases. The cleavage of PBP3A by CtpA is depicted in figure 9.7A. The chromatogram shows several peaks. The substrate peak could be found at 18.2 min. with a mass of 3441 (see mass spectrum in fig. 9.7B). The peak at 19.5 min. had a mass of 723 as can be seen in fig. 9.7C which corresponds with the substrate of the FTIC-Acp-MA with mass 723. The counter product could be indentified at the peak 11.7 min. The mass spectrum as depicted in figure 9.7D identified a fragment with mass 2736 (GSLRALAIPPDNLQDSPAVADRQHHG). The rest of the peak were peak already present in the substrate as impurities. Prc was also able to cleave the PBP3A peptide substrate. The LC-MS results are shown in figure 9.8. The chromatogram reveals two large peaks at 18.7 and 19.5 min. The peak at 18.7 min had a mass of 1207 as can be seen in the mass spectrum (see fig. 9.8C). The mass corresponds with cleavage product of FTIC-Acp-
MAGSLRA. The counter cleavage product could be found at the peak at 10.7 min. with a mass of 2251 (LAIPPDLQDSPA/VADRQHHG) (see fig. 9.8E). The second largest peak at 19.5 had the same mass spectrum as seen with the CtpA cleavage with a mass of 723 corresponding fragment FTIC-Acp-MA (see fig. 9.8D). The resulting counter cleavage product could be identified at 11.7 min. The mass spectrum showed a mass of 2736 corresponding a peptide with sequence GSLRALAIPPDLQDSPA/VADRQHHG (see fig. 9.8F). The substrate could be identified at peak 18.2 min with a mass of 3441 as shown in the mass spectrum in figure 9.8B. Prc from E.coli was also able to cleave the PBP3A substrate as shown before. The LC-MS results are shown in figure 9.9. The chromatogram shown a overall same result as the cleavage by CtpA. The largest peak at 18.2 had a mass of 3441 corresponding again with the substrate (see mass spectrum fig. 9.9B). The peak at 19.5 could be identified a cleavage product FITC-Acp-MA. The mass spectrum had a mass of 723 (see fig. 9.9C). The counter cleavage product GSLRALAIPPDLQDSPA/VADRQHHG could be identified at 11.7 min with a mass of 2736 (see mass spectrum fig. 9.9D).
Figure 9.4: Cleavage of PBP3 synthetic peptides with CtpA, Prc from *P. aeruginosa* and Prc from *E. coli*. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 9.5: Cleavage of PBP3A synthetic peptides with CtpA, Prc from *P. aeruginosa* and Prc from *E. coli*. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 9.6: Cleavage of PBP3 synthetic peptides with CtpA from *P. aeruginosa* by LC-MS. A) Chromatogram of the TIC. B) Mass spectrum of peak at 19.2 min. C.) Mass spectrum of peak at 12.4 min.
Figure 9.6 continuation: Cleavage of PBP3 synthetic peptide with CtpA from *P. aeruginosa* by LC-MS. D) Mass spectrum of peak at 19.5 min.

A: PBP3A with CtpA

B: FTIC-Acp-MAGSLRALAIPPDDLQLDPAVADRQHHG (MW3441) $[^{3}H][M+3H]^{3+}$

Figure 9.7: Cleavage of PBP3A synthetic peptide with CtpA from *P. aeruginosa* by LC-MS. A) Chromatogram of the TIC. B) Mass spectrum of peak at 18.2 min.
Figure 7 continuation: Cleavage of PBP3A synthetic peptide with CtpA from *P. aeruginosa* by LC-MS. C) Mass spectrum of peak at 19.5 min. D) Mass spectrum of peak at 11.7 min.

Figure 9.8: Cleavage of PBP3A synthetic peptide with Prc from *P. aeruginosa* by LC-MS. A) Chromatogram of the TIC.
Figure 9.8 continuation: Cleavage of PBP3A synthetic peptide with Prc from *P. aeruginosa* by LC-MS. B) Mass spectrum of peak at 18.2 min. C) Mass spectrum of peak at 19.5 min. D) Mass spectrum of peak at 11.7 min.
Figure 8 continuation: Cleavage of PBP3A synthetic peptide with Prc from *P. aeruginosa* by LC-MS. E) Mass spectrum of peak at 18.7 min. F) Mass spectrum of peak at 10.7 min.

A) PBP3 with Prc from *E. coli*

Figure 9.9: Cleavage of PBP3A synthetic peptide with Prc from *E. coli* by LC-MS. A) Chromatogram of the TIC.
Figure 9.9 continuation: Cleavage of PBP3A synthetic peptides with Prc from *E.coli* by LC-MS. B) Mass spectrum of peak at 18.2 min. C) Mass spectrum of peak at 19.5 min. D) Mass spectrum of peak at 11.7 min.
In order to validate the cleavage also a PBP3 peptide from *E.coli* was examined. These results are shown in **Figure 9.10**. The unprocessed substrate is depicted in **Figure 9.10A** and has a retention time of 18.4 min. The assay of CtpA and Prc are depicted in **Figures 9.10B and 9.10C**. Neither CtpA or Prc form *P.aeruginosa* were able to cleavage the PBP3 peptide of *E.coli*. Prc from *E.coli* on the other hand was able to cleave the PBP3 peptide. The assay resulted in one cleavage product with a retention time of 19.6 min. (see **fig. 9.10D**). The cleavage assay of the Prc from *E.coli* and the PBP3 substrate from the same organisme was further analysed by LC-MS. The results of this experiment can be seen in **Figure 9.11**. **Figure 11A** shows the chromatogram of the assay. The chromatogram shows two distinct cleavage peaks at 17.8 min. and 19.3 min. The corresponding mass spectrum at 17.8 revealed a mass of 3555 which indentifies the peak as the unprocessed substrate. The mass spectrum at 19.3 min. shows a mass of 2496 which corresponds to FITC-Acp-TMNIEPDALTGTGKNEFV.

The overall cleavage sites indentified are summarized schematically in **Figure 9.12**. CtpA showed cleavage ability to both PBP3A and PBP3. In both cases the cleavage was between an alanine and glycine, respectively between the 26-27th of PBP3 and 32-33th amino acid from the C-terminal of the PBP3A peptide. Prc of *P.aeruginosa* cleaves only PBP3A between the 26 and 27th (Ala-Gly) and 20 and 21th amino acid (Ala-Leu) from the C-terminus in almost equal preference to both cleavage sites. No cleavage of Prc was detected for the PBP3peptide. CtpA and Prc of *P.aeruginosa* showed no cleavage activity toward the PBP3 peptide of *E.coli*. The Prc from *E.coli* also cleavages both the *P.aeruginosa* PBP3 and 3A at the same cleavage sites as CtpA does. Prc of *E.coli* on the other hand cleaved the PBP3 from its own organism 11 amino acids from the C-terminal (between 11th and 12th amino acids) and hydrolyzed the bond between a valine and isoleucine.

The results show that both CtpA and Prc proteases are able to process the C-terminal of the PBP peptides. Although these assays are only based on short artificial peptides, these results give strong evidence that the PBP3 and PBP3A are substrates for at least one of these proteases. PBP3A is cleaved by both CtpA and Prc. PBP3 was only cleaved by CptA and not by Prc. The cleavage site of both CtpA and Prc for the PBP3A peptide was between the Ala-Gly 26th and 27th amino acid from the C-terminus. Prc had a second cleavage site between Ala-Leu at the 20th and 21th amino acid. Both cleavage sites are between alanine and a hydrophobic amino acid. It is known that CTPs preferably cleave within a alanine at the N-terminus side and
a hydrophobic amino acid on the C-terminus side or between two hydrophobic amino acids (Keiler et al., 1996). Based on the suggested cleavage site of the Prc from E.coli and the multiple alignment the predicated cleavage site of the PBP3 and PBP3A of P. aeruginosa lies much closer to the C-terminus than experimentally found. Based on the alignment of PBP3 between P. aeruginosa and E.coli the predicted cleavage side would between Glu^{12th}.Val^{11th} from the C-terminus. The glutamine is a hydrophilic amino acid with a amide group what would be unpreferable for the CTPs. Or with the knowledge about the preference of cleavage after a alanine between Ala^{9th}.Ala^{8th}. For the PBP3A peptide this would be between the Ala^{9th}.Val^{8th}. It could be that the peptide is cleaved consecutively. This is also found with the CtpA of cyanobacterium Synechocystis sp. PCC 6803 and cleavage of the pD1 protein (Inagaki et al., 2001). Here the C-terminal is processed twice. Although this could be possible no corresponding peptide fragments were found within the LC-MS analysis. It is interesting that Prc was unable to cleave the PBP3 as there is a high sequence similarity between PBP3 and PBP3A. The PBP3A peptide is 6 amino acids shorter than the PBP3 peptide. Remarkably enough Prc of E.coli was able to cleave both the PBP3 and PBP3A peptide.

There is a high sequence similarity between the Prc of P. aeruginosa and the proteases from E.coli. Prc of E.coli had the same cleavage sites as the CtpA for both PBP3A and PBP3. With PBP3 Prc for E.coli had an additional cleavage site between the 20th and 21th amino acid (Ala-Leu) from the C-terminus. The Prc of E.coli was able to cleave the PBP3 peptide of E.coli. The cleave site between the 12th and 11th from the C-terminus corresponds exactly with the cleavage site suggested by Nagasawa and co-workers (Nagasawa et al., 1989). This result confirms by an cleavage assay that the Prc directly processes the PBP3 C-terminus. Besides that this result makes the cleavage assay more valid as the artificial nature of the assay confirms the hypothesis.
Figure 9.10: Cleavage of PBP3 synthetic peptide from *E.coli* with CtpA, Prc from *P.aeruginosa* and Prc from *E.coli*. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 9.11: Cleavage of PBP3A synthetic peptide from E.coli with Prc from E.coli by LC-MS. A) Chromatogram of the TIC. B) Mass spectrum of peak at 17.8 min. C) Mass spectrum of peak at 19.3 min.
Figure 9.11 continuation: Cleavage of PBP3A synthetic peptide from *E. coli* with Prc from *E. coli* by LC-MS. D) Mass spectrum of peak at 4.8 min.

Figure 9.12: Cleavage site determination of the PBP3 peptide substrates. The cleavage site were determined by LC-MS. Cleavage site are marked with an arrow between two amino acids. Penicillin Binding Protein 3 and 3A from *P. aeruginosa* (PBP3-Pa, PBP3A-Pa) and Penicilline Binding Protein 3 from *E. coli* (PBP3-Ec).
PBP preferences

PBP3A is cleaved by both CtpA and Prc. To determine which CTP is more specific towards the PBP3A peptide the relative activities of both proteases were measured under standardised conditions. The relative activities are shown in figure 9.13. As in coherence with before PBP3 is only cleaved by CtpA and not by Prc. Both Prc and CtpA are able to cleave PBP3A. With a relative activity of 94.7% (SD +/- 7.7) Prc seems to be more active for PBP3A than CtpA is with a relative activity of 61.7% (SD +/- 6.1). It is interesting that the genome of P. aeruginosa has two similar CTP proteases CtpA and Prc where most bacteria have only one CTP. In search of a physiological substrate it was interesting to find two similar PBP3 proteins within the genome. One can therefore make the assumption that the two PBP3 proteins will be processed by the two individual CTP proteases. This is only partly true. The PBP3 is only being processed by CtpA. The PBP3A is being processed by CtpA as well as Prc. Although the relative activities towards the processing of PBP3A show that Prc has more activity towards the PBP3A compared to CtpA. In an important process of that of the septation of the cell both proteases may act as a back-up for each other. The assumption are made on the basis of artificial peptides and follow-up research has to be done for example in vivo to confirm these observations.
Figure 9.13: Relative protease activity of the Carboxy-terminus proteases from *P. aeruginosa* Prc (Prc-Pa), CtpA (CtpA-Pa) on the substrates Penicillin-binding protein 3 (PBP3-Pa) and 3A (PBP3A-Pa). Proteases (2 μM) were incubated overnight with substrate (20 mM) in 20 mM Tris and 200 mM KCl (pH 8.0) at 37 °C. Reaction were terminated by heat and subsequently analysed on HPLC.
CTP target isolation: physiological function of CtpA and Prc in *Pseudomonas aeruginosa*

**Cleavage of tmRNA peptide by CTPs**

Ribosomes can be stalled during the translation of a truncated mRNA. This could happen for example when a stop codon, that releases the mRNA from the ribosomes, isn’t present. These bacterial cells have a tmRNA system (initial named ssrA system) that have more or less three functions: 1) restore the stalled ribosome 2) disposal of wrong mRNA 3) tagging of the translated peptide toward degradation (Himeno et al., 2014). The system was suggested by Keiler and co-workers and showed that Prc of *E.coli* played are role in this system (Keiler et al., 1996). *Figure 9.14* schematically depicts the tmRNA system.

When ribosomes are stalled a alanine-tmRNA molecule binds in the A site of the ribosome (see *fig. 9.14A*). The alanine is bound to the residing peptide chain (see *fig. 14B*). The alanine-tmRNA moves to the P site in the ribosome and expels the defect mRNA (see *fig. 9.14C*). The tmRNA is now translated further by elongation of the peptide chain and is therefore tagged with the tmRNA sequence (see *fig. 9.14D*). This results in the synthesis of the defect protein with a tmRNA tag (see *fig. 9.14E*). The tagged protein is now directed towards degradation.

Keiler and co-workers showed that periplasmic proteins tagged with a tmRNA sequence were being degraded by Prc (Keiler et al., 1996). For *E.coli* the tmRNA tags result in a amino acids sequence of AANDENYALAA. This C-terminal hydrophobic peptide tag is being recognized by Prc and cleavaged.

Beebe and co-workers synthesized a artificial peptide Prc substrate for *E.coli* which contains the tmRNA sequence at its C-terminus (Beebe and Pei, 1998). Based on these results a similar peptide was design with the tmRNA sequence of *P.aeruginosa* and which was labelled with a N-terminal fluorescent dye. The tmRNA of *P.aeruginosa* is quiet similar to the *E.coli* tag (see *fig. 9.15A*). The structure of the tmRNA peptide substrate is depicted in *figure 9.15B*. 
Figure 9.14: Schematic representation of the tmRNA system. A) a stalled ribosome is recognized by alanine-tmRNA and binds to site A. B) alanine-tmRNA binds to the residing peptide chain. C) alanine-tmRNA moves to the P site and expels the defect mRNA. D) elongation of the peptide chain with the tmRNA reading frame. E) synthesis of the defect protein with tmRNA tag. F) Direction of tagged protein towards degradation.

**Figure 9.15:** A. Translated amino acid sequences of tmRNA tags. B. Chemical structure of the *P. aeruginosa* tmRNA peptide substrate.

<table>
<thead>
<tr>
<th>Tag</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>E.coli</td>
<td>ANDENYALAA</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>ANDDNYALAA</td>
</tr>
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**Figure 9.15:** A. Translated amino acid sequences of tmRNA tags. B. Chemical structure of the *P. aeruginosa* tmRNA peptide substrate.
CtpA and Prc were tested if the proteases were able to cleave this substrate. The results are shown in figure 9.16. The results show that CtpA and Prc both are able to cleave the tmRNA substrate (see fig. 9.16A and B). The hydrolysis of the substrate shows two degradation peaks at 17.3 and 16.4 min. The substrate peak is not present anymore. Prc from E.coli was also able to cleave the tmRNA peptide showing the same two degradation peaks and time as with the CtpA and Prc from P.aeruginosa. The Prc of E.coli has shown activity before toward a similar peptide but with the E.coli tmRNA sequence tag (Beebe, 1998). The tmRNA peptide in this experiment had the P.aeruginosa tmRNA sequence tag although there are very similar. The only difference in the tmRNA sequence between P.aeruginosa and E.coli is the appearance of a aspartic acid (D) instead of an glutamic acid (E) which have very similar chemical properties.

The experiments were followed up by determination of the cleavage sites by LC-MS. These results are also shown in figure 9.17-19. All CTPs cleaved the tmRNA peptide substrate at two distinct sites and the chromatogram and corresponding mass spectra are similar. The chromatogram show degradation peak at 17.7, 16.1, 10.7 and 10.2 minutes (see fig. 9.17-19A). The mass spectrum of the first peak shows a mass of 662 which corresponds with FTIC-Acp-AA (see fig. 9.17-19B). The N-terminal portion of this cleavage can be found at the peak at 10.2 min. with shows a mass of 1389 indentifying the portion of RAAK-Ahx-Ahx-DNYALLA (see fig. 9.17-19C). The second cleavage product at retention time 16.1 show in the mass spectrum a mass of 960 which reveals the C-terminal part of FTIC-Acp-AARAA (see fig. 9.17-19D). The corresponding N-terminal part can be found at the peak at 10.7 min which has in its mass spectrum a mass of 1090 which indicates K-Ahx-Ahx-DNYALLA (see fig. 9.17-19E). The major cleavage site is the hydrolysis between the Ala^{14th}-Arg^{13th} from the C-terminus, followed by the Ala^{11th}-Lys^{10th}. The major cleavage site between the Ala^{14th}-Arg^{13th} corresponds with the site found with the similar peptide substrate with the E.coli tmRNA tag (Beebe, 1998). The indentified cleavage sites are summarized in figure 9.20.
CtpA and Prc were able to recognize the tmRNA peptide sequence and cleavage the peptide. These results shown that both CTPs may play a role in the degradation of tmRNA tagged periplasmic proteases. These results are preliminary and should be investigated further. Although these observations confirm the results of Prc from *E.coli* which is shown to play a role in the tmRNA tagging system. The CTP will not be the only proteases involved in this system as CTP will not be able to degrade the peptides of protein fully.
Figure 9.16: Cleavage of the tmRNA synthetic peptide with CtpA, Prc from *P. aeruginosa* and Prc from *E. coli*. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 9.17: Cleavage of the tmRNA synthetic peptide with CtpA from *P. aeruginosa*. A) Chromatogram of the TIC. B) Mass spectrum of peak at 17.7 min. C) Mass spectrum of peak at 10.2 min.
Figure 9.17 continuation: Cleavage of the tmRNA synthetic peptide with CtpA from *P. aeruginosa* by LC-MS. D) Mass spectrum of peak at 16.1 min. E) Mass spectrum of peak at 10.7 min.
Figure 9.18: Cleavage of the tmRNA synthetic peptide with Prc from *P. aeruginosa* by LC-MS.

A) Chromatogram of the TIC. B) Mass spectrum of peak at 17.7 min. C) Mass spectrum of peak at 10.2 min.
Figure 9.18 continuation: Cleavage of the tmRNA synthetic peptide with Prc from P. aeruginosa by LC-MS. D) Mass spectrum of peak at 16.1 min. E) Mass spectrum of peak at 10.7 min.
Figure 9.19: Cleavage of the tmRNA synthetic peptide with CtpA, Prc from *P. aeruginosa* and Prc from *E. coli* by LC-MS. A) Chromatogram of the TIC. B) Mass spectrum of peak at 17.7 min. C) Mass spectrum of peak at 10.2 min.
CTP target isolation: physiological function of CtpA and Prc in *Pseudomonas aeruginosa*

Figure 9.19 continuation: Cleavage of the tmRNA synthetic peptide with Prc from *E. coli* by LC-MS. C) Mass spectrum of peak at 16.1 min. C) Mass spectrum of peak at 10.7 min.

Figure 9.20: Cleavage site determination of the tmRNA peptide of the CtpA and Prc of *P. aeruginosa* (Pa) and the Prc of *E. coli* (Ec). Proteases (2 μM) where incubated with substrate (20 mM) in 20 mM Tris and 200 mM KCl (pH 8.0) at 37 °C. Reaction were terminated by heat and subsequently analysed by LC-MS.
Chapter Discussion

The physiological substrate of bacterial CTP proteases are yet unknown although there are some suggestions. One of the best investigated substrates are Penicillin Binding Proteins. CtpA from *P. aeruginosa* was able to cleave PBP3 and PBP3A peptide substrates. Hereby CtpA seems to have an more preference towards the cleavage of PBP3 as shown by a faster relative activity. Prc was only able to process the PBP3A. These results confirm the hypothesis that PBP3 can be processed by CTPs. It would make sense that if an organism has two distinct PBP3 proteins that it has to have two proteases that can process the proteins. The results show that there is no clear specificity towards one PBP3. Only Prc specifically processes the PBP3A and not the PBP3. But CtpA is able to cleave both protein. This difference in cleavage pattern may be involved in the complex mechanisms involved in the septation process. It seems more unlikely that the genome of *P. aeruginosa* would contain two proteases with exact the same function. If PBP3 and PBP3A are physiological substrates for CtpA and Prc needs to be investigated and confirmed by *in vivo* experiments.

Besides that the results show that Prc from *E. coli* can directly process the PBP3 from its own organism. This assay has never been executed before and they confirm early observations that Prc possibly cleavages PBP3. More strikingly Prc cleavages exact at the same site as was investigated in prc negative mutants. This makes the observations made about the *P. aeruginosa* proteases more valid. Besides that if PBP3 are confirmed to be physiological substrates of CTPs this would also explain their highly conserved presence in almost every bacterial genome known.

As PBP3 proteins are probably involved in cell wall biosynthesis and septum formation these processes could explain the earlier observation made in Chapters 5 and 6. Highly speculative, inactivation of the CTPs in *P. aeruginosa* would disturb or disrupt cell wall synthesis and septum formation. It is plausible that a malfunction in one of these processes could lead to a defect in the secretion of proteins and virulence factors. And this could lead to an decrease in establishing an infection which was observed in the different infection models.

Keiler et al. suggested CTPs could also be involved in the tmRNA tagging system. This system tags incorrectly synthesized protein in the ribosomes and directs them for degradation. Both CtpA and Prc were able to cleavage a tmRNA specific design peptide substrate. These results
suggests that CTPs could be involved in multiple cellular processes such as the tmRNA system. Again as with the PBP substrates these are artificial tmRNA peptides which mimic the C-terminal of proteins. The involvement of CtpA and Prc in the system has to be investigated in more detail.
References


CTP target isolation: physiological function of CtpA and Prc in *Pseudomonas aeruginosa*
"System recovery may prove impossible"

IAN MALCOLM

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff
CHAPTER 10
Results and discussion

CTP target characterization:

substrates from green organisms and 3D modeling

This chapter is partly based on:

Hoge RHL, Gironés D, Laschinski M, Wirtz A, Santiago B, Nabuurs SB, Jäger KE, Rosenau F.
Activity and cleavage mechanism elucidation of a C-terminal processing protease from
Chapter summary

Bacterial CTPs show high similarities to CTPs from chlorophyll-b containing green organisms. Cleavage experiments with synthetic peptide substrates mimicking the C-terminal of the pD1 protein of Spinacia oleracea (Spinach) and S. obliquus obliquus (green algae) were executed. Results show that CtpA and Prc of P. aeruginosa as well as the Prc of E. coli can hydrolyse these peptides. The cleavage site of these peptides were in line with the known cleavage sites observed with the native CTPs of these organism. These data confirm the high evolutionary resemblances of these proteases.

3D modeling studies were executed in order to understand the mechanism of cleavage of these proteases. 3D modeling of the PDZ domain of S. obliquus bound with a substrate peptide revealed several characteristics that could explain the peptide hydrolysis mechanism of CTP proteases. First a proline at position P-4 enables a turn within the substrate peptide necessary for binding at the PDZ domain and the active site. Secondly a threonine (Thr168) interacts with the residue at position P-3 which is therefore possibly necessary for specificity. Thirdly the structure reveals regions at the beginning and end of the PDZ domain which could perform a hinge motion. This could facilitate an open and closed conformation of the protease enabling variable substrate peptides lengths. A fourth characteristic would be the identification of a hydrophobic path in the active site interacting with conserved peptides at positions P1-P4 of the substrate enabling specificity.

The results of this chapter were obtained in collaboration with:

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Centre for Molecular and Biomolecular Informatics,
Radboud University Medical Centre, Nijmegen, the Netherlands

LeadPharma BV, Nijmegen, the Netherlands

The 3D modeling experiments and cleavage mechanism elucidations were executed by Daniel Gironés.
Cleavage of D1 peptides by bacterial CTPs

Phototrophic organisms such as plants, algae and cyanobacteria have a CtpA protease encoded within their genomes. The CtpA plays its role in the activation of the D1 precursor protein which is part of Photosystem II. The function of the CtpA is explained in figure 10.1. Photosystem II, which is a complex of more than 20 proteins, contains two core proteins D1 and D2. The gene of the D1 protein is encoded in the chloroplastic DNA as precursor protein pD1. mRNA of pD1 is translated in the stroma and translocated over the thylakoid membrane. The pD1 is directly folded and integrated in the thylakoid membrane as a precursor. The CtpA protease is encoded in the genomic DNA and translated and folded within the cytoplasm.

The CtpA protease contains two N-terminal signals, a stromal-import sequence and a signal sequence for translocation across the thylakoid membrane into the thylakoid lumen. The protease is active within the thylakoid membrane and cleaves the pD1 into its mature form. After cleavage the D1 protein contains a C-terminal alanine which is necessary for binding a Mn₄Ca-cluster. Mn₄Ca-clusters form part of the functional Photosystem II as they are responsible for water oxidation (Satoh and Yamamoto, 2007).

Bacterial CPTs are similar to the CtpA from chlorophyll-b containing green organisms. Figure 10.2 shows a multiple alignment analysis with two phototrophic organisms, Spinacia oleracea (Spinach) and S.obliquus obliquus (green algae), and the three CTPs from bacteria P.aeruginosa and E.coli. The alignment shows the two function domains of CTPs in both bacterial and phototrophic organisms.
Figure 10.1: Schematic representation of the function of CtpA within a phototrophic organism. A detailed description is found in the text below. Briefly, CtpA is encoded within the genomic DNA, translated and synthesized within the cytoplasm. The CtpA is translocated to the stroma and subsequently to the thylakoid lumen. In the thylakoid lumen CtpA processes the preD1 protein by C-terminal cleavage forming the active D1 protein (Satoh and Yamamoto, 2007).
CTP target characterization: substrates from green organisms and 3D modeling
Figure 10.2: Multiple alignment of CTPs. S41 subfamilies are designated CTP1, CTP2 and CTP3. CtpA *Spinacia oleracea* (Spo; BAA09134.1); CtpA *S.obliquus obliquus* (Sco; O04073.1) CtpA and Prc *Pseudomonas aeruginosa* (Pa; PA5134; PA3257); Prc *Escherichia coli* (Ec; M75634.1).
Because of this resemblance between bacterial and CTPs from phototropic organisms it is interesting to see if the bacterial proteases are able to process substrates from plants or green algae. Therefore two D1 peptides were design for the organisms Spinacia oleracea (Spinach) and S.obliquus obliquus. The substrates are depicted in figure 10.3. In the figure is also shown what the native cleavage site are of these organisms. The pD1 from Spinach and S.obliquus are cleaved 9 amino acids from their C-termini. Additional the pD1 from S.obliquus can also be cleaved 5 amino acids from the C-terminus (Satoh and Yamamoto, 2007).

Figures 10.4 and 10.5 show the results of the cleavage experiment with CtpA and Prc from P.aeruginosa and Prc from E.coli. CtpA and Prc of P.aeruginosa were able to cleave both D1 peptide substrates of Spinach and S.obliquus. CtpA and Prc showed a same degradation profile. The chromatogram shows one distinctive cleavage peak (19.7 min.) with a longer retention time as the substrate peak (19.2 min.) and a minor cleavage peak with a short retention time at 18.3 min. Prc from E.coli was also able to cleave both substrates. In the chromatograms three cleavage products peaks can be seen. Two peaks have the same retention time as the degradation peaks of CtpA and Prc at 19.7 and 18.3 min. Additional a major degradation peak can be seen at 17.3 min. with a much shorter retention time as the substrate peak with both the D1 from Spinach as from S.obliquus.

Subsequently the cleavage sites of all three CTP proteases were determined by LC-MS. The results of this analysis are depicted in figures 10.6 and 10.7. CtpA and Prc cleaved both the D1 peptide substrates between the same amino acids as their native CtpAAs. For Spinach is the D1 peptide substrates cleaved between the Ala^{10th}-Ala^{9th} amino acids from the C-terminus. The D1 peptide substrate of S.obliquus is being cleaved between the Ala^{10th} and Ser^{9th} amino acids form the C-terminus. Here again the hydrolysis takes place between a alanine at the N-terminus site and a amino acid with a small side chain such as alanine or serine on the C-terminus of the cleavage site.

Prc from E.coli also cleavages both the D1 peptide substrates at this same cleavage site but also at an additional site. The predominant cleavage site is the same at the native cleavage sites of Spinach and S.obliquus. The sequence of the additional site lies between Ala^{18th} and His^{17th} from the C-terminus of both the Spinach substrate as the substrate from S.obliquus.
Chapter 10

Alanine is the second and last amino acid form the C-terminus of both D1 peptide substrates. In addition to the cleavage site also a PSVNA fragment was found in the chromatograms of the D1-SEO substrate with all proteases. For *S. obliquus* cleavage of the pD1 protein has been suggested to take part in two steps. First the cleavage of 5 amino acids (PSVNA) and after that an additional 4 amino acids in total 9 amino acids from the C-terminal. The cleavage site of the peptides are schematically summarized in figure 10.8.
Figure 10.3: A. D1 protein cleavage sites of the native CtpA from *Spinacia oleracea* and *S. obliquus obliquus*. B. Chemical structures of the D1 fluorescent peptide substrates.
Figure 10.4: Cleavage of D1 synthetic peptide from *S.oleracea* with CtpA, Prc from *P.aeruginosa* and Prc from *E.coli*. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 10.5: Cleavage of D1 synthetic peptide from \textit{S.obliquus} with CtpA, Prc from \textit{P.aeruginosa} and Prc from \textit{E.coli}. HPLC analysis with fluorescence detection (Ex: 494nm, Em: 518nm).
Figure 10.6: Cleavage of D1 S.oleracea synthetic peptide by bacterial CTPs by LC-MS. A) Chromatogram of the substrate without protease B) Chromatogram of the CtpA cleavage. C) Chromatogram of the Prc from P.aeruginosa cleavage D) Chromatogram of the Prc from E.coli cleavage
**Figure 10.6 continuation: Cleavage of D1 S.oleracea synthetic peptide by bacterial CTPs by LC-MS.** Representative Mass spectra E) Mass spectrum of peak at 18.0 min. F) Mass spectrum of peak at 18.3 min. G) Mass spectrum of peak at 7.7 min.
Figure 10.7: Cleavage of D1 S. obliquus synthetic peptide by bacterial CTPs by LC-MS. A) Chromatogram of the substrate without protease B) Chromatogram of the CtpA cleavage. C) Chromatogram of the Prc from P. aeruginosa cleavage D) Chromatogram of the Prc from E. coli cleavage
Figure 10.7 continuation: Cleavage of D1 S. obliquus synthetic peptide by bacterial CTPs by LC-MS. Representative Mass spectra A) Mass spectrum of peak at 18.3 min. B) Mass spectrum of peak at 8.9 min. D) Mass spectrum of peak at 16.3 min.
Figure 10.8: Cleavage site determination of the pD1 peptide substrates of the CtpA and Prc of \textit{P. aeruginosa} (Pa) and the Prc of \textit{E. coli} (Ec). Proteases (2 µM) were incubated with substrate (20 mM) in 20 mM Tris and 200 mM KCl (pH 8.0) at 37 °C. Reaction were terminated by heat and subsequently analysed by LC-MS.
3D modeling

In order to understand the mechanism of the peptide cleave in silico experiments were started to obtain high quality 3D models of bacterial CTPs. Until now only one CTP protease structure has been elucidated by X-ray crystallography, namely CtpA of Scenedesmus obliquus. This CtpA is involved in the same photosynthesis process as the CtpA of Spinacia oleracea and S. obliquus obliquus.

The sequence of the CtpA from S. obliquus contains 427 amino acid residues. A pairwise sequence alignment with the CtpA from P. aeruginosa shows 32% identity and 48% similarity. In Synechocystis the carboxyl-terminal extension in pD1 is 16 amino acid residues long (Nixon et al., 1992). Figure 10.9 shows the solved structure of CtpA from S. obliquus. The three different domains are indicated in the figure with different colours.

Inagaki et al. identified 14 conserved residues based on multiple alignments of members of this protease family and subjected them to site-specific mutations. Their research showed that five of these residues were critical for the in vivo processing of pD1, namely Asp253, Arg255, Ser313, Glu316 and Lys338 (*, figure 10.10) (Inagaki et al., 2001). Multiple sequence alignment with the sequences of S. obliquus and P. aeruginosa shows the equivalent residues (orange boxes, figure 10.10). The sequence similarity is greater in the C domain, where the active site is located.
Figure 10.9: Solved structure by X-ray crystallography of CtpA from *S. obliquus*. Domains are indicated with different colours: N-terminal domain (A; red), PDZ domain (B; yellow), catalytic domain (C; blue).
Figure 10.10 Multiple sequence alignment of CtpA from *Synechocystis* sp. PCC 6803 (Syn-CtpA), *S.obliquus* (Sco-CtpA) and *P.aeruginosa* (Pa-CtpA). The coloured bars represent the different domains according to the 3D structure of *Synechocystis* (fig.10.9). The five critical residues are indicated with the *.* Orange boxes represent identical residues within the alignment.
To have a better insight on the binding mode of the substrate and the cleavage mechanism of CtpAs we decided to model the B domain of *S. obliquus* CtpA with a bound peptide substrate and replace it in the crystal structure.

Generally, PDZ domains recognize C-terminal motives of five residues in length. These ligands bind as a β-strand which adds to the edge of the β-sheet within the PDZ domain. This mechanism is known as β-strand addition (Harrison et al., 1996). The C-terminal residue is named the P₀ residue, and subsequent residues toward the N-terminus are referred as P₋₁, P₋₂, P₋₃, etc. (Harris and Lim, 2001). Studies suggest that the most critical ligand residues for recognition are P₀ and P₋₂. The PDZ domains are divided into three main classes depending on their preference for residues at these two positions (Songyang et al., 1997). The C-terminal residues of the pD1 processed by *S. obliquus* CtpA are "PSVDA". Based on this sequence the PDZ domain of *S. obliquus* CtpA falls on class II, which recognizes the motive ϕ-X-ϕ-CO₂H, where ϕ is a hydrophobic amino acid and X is any amino acid.

The typical secondary structure topology of a PDZ domain consists of 6 β-strands (βA-βF) and 2 α-helices (αA and αB) as depicted in figure 10.11A. Interestingly, the crystal structure of *S. obliquus* CtpA displays a PDZ domain with a circular permutation when compared to the canonical PDZ domains (figure 10.11B) (Liao et al., 2000).

In order to generate a good model of the PDZ domain of *S. obliquus* CtpA five high resolution crystal structures of PDZ domains bound to a substrate segment were identified within the PDB database, where the peptide substrate varies between four and six amino acid residues (figure 10.12).

The secondary structure of these five PDZ domains is represented by the canonical form depicted in figure 10.11A. To be able to utilize these structures as templates their sequence connectivity was modified by linking the N and C-termini and disconnecting the β-strands βA and βB. The modified PDZ domains display a new amino acid sequence matching the secondary structure found in *S. obliquus* CtpA (figure 10.11B).
Figure 10.11: Secondary structure topology of PDZ domains. A) topology of PDZ domains in general. B) topology of the PDZ domain of S. obliquus CtpA.

Figure 10.12: Five crystal structures of template PDZ domains. Substrate peptides are colored in yellow.
A multiple sequence alignment was performed between the template sequences and the target, which contained the C-terminal segment of its known endogenous substrate. A model of the PDZ domain with a five amino acid bound peptide was generated and replaced in the crystal structure between Val163 and Leu245. Energy minimization of the whole system yielded a modelled structure of *S. obliquus* CtpA with a 5 amino acid residue bound peptide which is depicted in figure 10.13.

The P₄ proline residue is highly conserved in pD1 among different species (Satoh and Yamamoto, 2007). It does not show a direct relevant interaction with the PDZ domain but we observe that it promotes a turn, thus adapting the rest of the ligand to the CtpA surface and over the Ser/Lys dyad active site. Jansèn *et al.* speculated that Thr168 might play a role in optimal catalytic activity (Jansen, 2003). Indeed the *S. obliquus* CtpA crystal structure shows Thr168 Oγ within hydrogen bond distance to Lys397 Nζ. However, in the model, Thr168 Oγ shows a hydrogen bond with the P₃ serine residue in the substrate, indicating a role in substrate recognition specificity.

The modeled *S. obliquus* CtpA structure shows the PDZ domain too close to the active site to be able to accommodate a peptide sequence of 9 or 16 amino acid residues. Liao *et al.* suggested that the C-terminal sequence could be accommodated by making a loop (Liao, 2000). Regarding the similar CtpA of *Synechocystis*, Inagaki *et al.* have proposed a two-step proteolytic process of the substrate (Inagaki *et al.*, 2001). However, we are of the opinion that the PDZ domain can adopt a different conformation respect the rest of the enzyme, thus being able to accommodate C-terminal peptides of different lengths. In the crystal structure, the high Debye-Waller factor in the PDZ connecting loop residues suggests that they could perform a hinge motion, thus moving away the PDZ domain and bringing the scissile bond next to the active site. To study this possible mechanism a model of the *S. obliquus* CtpA was build with a 16-residue native substrate peptide where the cleavage product contains nine residues: NFPLDLA-SVEAPSVNA. The model was constructed with two different PDZ conformations considering a closed and open hinge (figure 10.14).
Figure 10.13: Model of *S. obliquus* CtpA containing a segment of the C-terminal sequence of its pD1 substrate "Ac-PSVDA".

Figure 10.14: Models of the *S. obliquus* CtpA with a 16-residue native substrate peptide showing the position of the scissile bond respect to the active site. A) The PDZ domain is positioned as in the crystal structure. B) Open hinge motion of the PDZ domain to accommodate the substrate.
Independently of the chain length on the C-terminal extension it is well established that the sequence upstream of the scissile bond is highly conserved among different autotrophic species. The amino acid residues P1-P4 (Schechter and Berger nomenclature) correspond to “LDLA”, suggesting that the substrate specificity is not only determined by the interaction of the PDZ domain with the C-terminal sequence of the substrate (Satoh and Yamamoto, 2007). Other relevant factor is the hydrophobic patch (S1-S4) next to the active site, which is able to recognize the LDLA sequence. Liao et al. have also consider the possibility of a substrate assisted catalysis making the pD1 Asp342 (P3) a third member of a catalytic triad (Liao et al., 2000). However, in the model the distance between pD1 Asp342 Oδ and Lys397 Nζ is more than 10 Å, which is too large for Asp342 to play any role in the catalysis. In the contrary, our model shows that pD1 Asp342 Oδ makes a hydrogen bond with S. obliquus CtpA Thr325 Oγ (figure 10.15). The equivalent residues in Synechocystis and P. aeruginosa are Ser343 and Thr332 respectively, suggesting another factor in substrate recognition.
Figure 10.15 Substrate segment showing the residue side chains “LDPAS” over the active side and the adjacent hydrophobic patch of *S. obliquus* CtpA. Note the hydrogen bond with Thr325.
Homology model of *Pseudomonas aeruginosa* CtpA

In order to understand the mechanisms involved in the CTPs of *P. aeruginosa* a structural model of CtpA was constructed based on the amino acid sequence alignment and the crystal structure of the *S. obliquus* CtpA (Figure 10.16). The folds of *P. Aeruginosa* CtpA homology model showed a high similarity with the *S. obliquus* CtpA tertiary structure. The low number of conserved amino acids at the beginning of the A domain and at the end of the C domain (N and C-terminal parts, see sequence alignment in Figure 10.10) make these areas less reliable in the model. Nonetheless, the higher number of conserved amino acids at the end of the A domain, the PDZ domain (B domain) and around the active site (C domain) validate well the model especially in terms of mechanistic activity.

Domain A (Figure 10.16) is composed of a bundle of three helixes followed by a short β-strand and a short fourth helix. Helixes one and two appear to be connected by a large loop, although this region of the model is less reliable. Domain B (Figure 10.16) functions as a PDZ domain, which displays the uncommon connectivity described for *S. obliquus* CtpA. Thus, the secondary structure matches the topology depicted in Figure 10.11B. Domain C (Figure 10.16) contains the active site next to which a hydrophobic patch can be found. It consists of three helixes fringed by two twisted β-sheets. A fourth short helix is located at the C-terminus directly interacting with the first β-strands.

Domain A

Liao et al. described a small cluster of five cationic residues located in the N-terminal domain as a possible membrane recognition site, since pD1 containing the C-terminal substrate is an integral membrane protein (Liao et al., 2000). A model of *Synechocystis* CtpA also showed a positively charged area in the domain A (Jansen et al., 2003). However, *P. aeruginosa* CtpA contains only two arginines in the same region (Arg47 and Arg55). This might be linked with the evolutionary change in the yet unknown function of non-photosynthetic CtpAs.
Figure 10.16 Model of CtpA from *P. aeruginosa* covering residues T9-A393.

Figure 10.17 Central area of the model of *P. aeruginosa* CtpA showing the PDZ domain bound to the short peptide “Ac-SVDA” and the active site region.
PDZ domain

A second model was generated of *P. aeruginosa* CtpA containing a segment of a C-terminal substrate. However, such substrate sequence is yet unknown. In view of the similarities between *S. obliquus* and *P. aeruginosa* CtpAs a four residue segment of *S. obliquus* CtpA substrate was chosen (Ac-SVDA). The same methodology was applied as before and the 5 crystal structures of PDZ domains containing a ligand peptide were used as templates (figure 10.12). The alignment of their sequences with modified connectivity (figure 10.11) against the PDZ domain of *P. aeruginosa* CtpA was utilized to generate the new model (figure 10.17). In this case the serine Oγ belonging to the substrate does not show a hydrogen bond with the PDZ domain, since there is a glycine (Gly112) instead of a threonine at the equivalent position compared to *S. obliquus* CtpA (Thr168, figure 10.13). Nevertheless, the rest of the ligand residues make a perfect match with the C-terminal recognition site.

Domain C and active site

The domain C contains the five conserved residues which are essential for activity, namely Asp240, Arg242, Glu305 and active site Ser302/Lys327 (Inagaki et al., 2001). Although located in the vicinity of the active site, Glu305 cannot play a role in a possible catalytic triad. Glu305 is in the same helix as Ser302, and the carboxyl group of its side chain is hydrogen bonded to the main chain amide nitrogens of Ser302, Gly326 and Lys327. It seems to play an important role in structural stability by bringing together Ser302 and Lys327. The distance between Ser302 Cα and Lys327 Cα is 4.8 Å.

Asp240 and Arg242 seem to also play a role in structural stability. A side view of the bottom part of domain C is depicted in figure 10.18. Arg242 makes a hydrogen bond with a loop connected to the short helix at the C-terminus, and Asp240 stabilizes the position of Arg242. Interestingly, Tyr205 is hydrogen bonded to Asp240 and also interacts with the hydrophobic inner side of the C-terminal helix. This tyrosine, although not identified as essential for the enzyme activity, is highly conserved among CtpAs. The interactions between these residues hold the C-terminal helix in a specific conformation, suggesting an important role in signaling or another yet unknown function for this helix.
Figure 10.18 Model of *P. aeruginosa* CtpA: side view of the bottom part of domain C
The CtpAs are considered serine proteases with a Ser/Lys catalytic dyad instead of the typical Ser/His/Asp catalytic triad observed in most serine proteases. But how can a lysine act as a general base in catalytic dyads? A deprotonated ζ-amino group is necessary for the lysine to act as a general base in catalytic dyads. A free lysine in water has a pKₐ of 10.5, thus there should be a local environment in the enzyme that enables a deprotonated state. A lower pKₐ value in a lysine can be produced by an electrostatic or polarity change in the environment (Paetzel and Dalbey, 1997).

From the electrostatic point of view, a lysine with a low pKₐ can be generated when a positive charge is in its vicinity, such as the presence of another lysine or an arginine. In the *P. aeruginosa* CtpA model the only proximal charged residue to the active site is Lys343. The average distance between the ζ-amino groups is 7.5 Å, which appears to be too far to generate any net change in the pKₐ of Lys327.

Regarding polarity, a hydrophobic microenvironment can induce a lowered lysine pKₐ. It is reported that a buried lysine can have a pKₐ as low as 6.5 (Dao-pin et al., 1991). In the structural studies of CtpAs most residues in the immediate vicinity of the lysine of the active site are hydrophobic, with exception of the catalytic serine. However, in an open conformation of the PDZ domain the active site would be too exposed to the surface. Generally, the residues adjacent to the scissile bond are hydrophobic. Therefore a hydrophobic microenvironment could be produced upon binding of the substrate inducing solvent exclusion (Liao et al., 2000). Under these conditions the lysine pKₐ can be lowered, allowing the serine hydroxyl and lysine amine to interact in a triple hydrogen bond arrangement generating an activated serine (figure 10.18) (Borman, 1995).
Figure 10.18 A) Ser/His/Asp canonical serine protease triad. B) Ser/Lys protease dyad.

Figure 10.19 Proposed mechanism in the catalytic cycle of *P. aeruginosa* CtpA.
*P. aeruginosa* CtpA also displays a hydrophobic patch next to the active site composed of Leu249, Val253, Leu277, Phe279, Val330 and Tyr349 (figure 10.18). When binding to the protease, the substrate should be in an extended conformation near the cleavage site suggesting that a short sequence of hydrophobic residues should be present in the substrate preceding the scissile bond. Interestingly, Thr332 (figure 10.16) is in an equivalent position as Thr325 from *S. obliquus* CtpA (figure 10.14) and could make a hydrogen bond with a substrate residue at P3 position. All these similarities made us consider that *P. aeruginosa* CtpA should be able to process the native C-terminal substrate from *S. obliquus* CtpA. This point was indeed corroborated experimentally as shown in figure 10.8. Upon binding of the substrate, the Oγ of the activated Ser302 performs a nucleophilic attack, where the electrophile is the trigonal planar carbonyl carbon taking part in the scissile bond. A tetrahedral intermediate transition state is formed, which it is possibly stabilized by the backbone amide nitrogens of Gly247 and Ala303 acting as an oxyanion hole. The tetrahedral intermediate rearranges to eliminate the C-terminal peptide. The remaining acyl-enzyme intermediate is attacked by an activated water molecule forming a second tetrahedral transition state, which again can be stabilized by the oxyanion hole. The rearrangement of the second transition state releases the processed substrate protein (figure 10.19).
**Chapter Discussion**

Remarkable that all the three bacterial proteases are able to cleave the D1 peptide substrates from a plant and a green algae at their same native cleavage site. As the bacterial CTPs are evolutionary connected with the CtpA from plants and green algae also their substrates could be similar. The physiological substrate of CtpA in plants, green algae and cyanobacteria is elucidated as being the pD1 protein. Inactivity of this CtpA is lethal to these organisms as a precursor D1 is non functional and as a result photosynthesis is impossible. Many bacteria such as *P.aeruginosa* and *E.coli* are not phototrophic and so they don’t have a Photosystem. Blast searches with the pD1 protein sequences in the genome of *P.aeruginosa* didn’t revealed a similar protein neither did Blast searches with only the C-terminal part of the pD1 protein.

In order to understand the mechanism involved in the hydrolysis of peptides with CTPs 3D modeling studies were executed. The modelled PDZ structure of CtpA from *S.obliquus* and that of *P.aeruginosa* showed both the same topology as described before by Liao et al. (Liao et al., 2000). A model experiment with the known C-terminal peptide of CtpA from *S.obliquus* was performed and revealed several key characteristics of the cleavage mechanism. First it seems that a proline residue at position P-4 is necessary to facilitate a turn which adapted the ligand to the CtpA surface and the Ser/Lys dyad active site. Secondly the model revealed a threonine Trh168 to be important for substrate recognition specificity as the residue interacts with the serine residue at P-3. Thirdly the PDZ seems to have two connecting loops which enables a hinge motion. This way the proteases could have different conformations, a closed or open form, which enables the protease to adjust itself depending on the length of the substrate. It is know that CtpA from *S.obliquus* can cleave at 9 and/or 16 amino acids from the C-terminus. Substrate binding on the PDZ domain and adjustment above the scissile bond within the active site would not be feasible in the closed conformation. A hinge motion could explain the allowance of larger substrate peptide in the protease. The forth characteristic is a hydrophobic path in the vicinity of P1-P4 of the substrate peptide. The sequences at positions P1-P4 corresponds to “LDLA” which could interact with the hydrophobic path of the active site. This would suggest a second specificity characteristic of the substrate within the active site besides the C-terminus of the substrate.
Modeling of the CtpA from *P. aeruginosa* revealed evidence that the proposed mechanism of a catalytic dyad Ser/Lys is more plausible than a Ser/His/Asp canonical triad common in serine proteases.
References


"System takeover seems to be inevitable"

IAN MALCOLM*

*Michael Crichton (1997), Jurassic Park, Amsterdam: Luitingh-Sijthoff
CHAPTER 11
Results and discussion

CTP target inhibition:

HTS assay and inhibitor screening

This chapter is partly based on:

The results of this chapter were obtained in collaboration with:

**Daniel Gironés and Sander Nabuurs**  
Centre for Molecular and Biomolecular Informatics,  
Radboud University Medical Centre, Nijmegen, the Netherlands

LeadPharma BV, Nijmegen, the Netherlands

The 3D modeling and docking experiments were executed by Daniel Gironés.
**Inhibitors of bacterial Carboxy-terminal processing proteases**

As stated in Chapter 1 development of new antibiotic drugs is necessary to win the combat from infectious diseases and the ever increasing problem of multi-drug resistance. Therefore the aim of this thesis was to identify a novel antibiogtarget. In Chapter 5 Carboxy-terminal processing proteases of *P. aeruginosa* were identified as possible targets. In the subsequent chapters these targets were validated in *in vivo* infection models, localized, cloned, expressed and purified. In Chapter 8 we showed that the proteases were active in a *in vitro* assay and followed by Chapter 9 in which was shown that the CTPs from *P. aeruginosa* were be able to cleave C-terminal peptide mimics of the Penicillin-binding protein 3 and 3A. We suggested that PBP3 and 3A may be the physiological substrates of these CTP proteases. Because the PBP3 proteins are involved in the cell wall synthesis of bacteria this could explain the shown secretion defect of virulence factors and the resulting attenuation of the pathogen.

The final aim of this thesis was to identify novel small molecular compounds that function as inhibitors for these proteases. The function of the inhibitor should be to inactivate the CTPs and therefore attenuated the bacterium. The attenuated bacterium is then disarmed of his pathogenesis and therefore less infective.

Although we presented in Chapter 8, 9 and 10 different activity assays these aren’t suitable for inhibitor screening. In order to find novel inhibitors that may be used as lead compounds in the drug discovery process, small chemical compound libraries are screened with typical amounts of 10,000 to 1,000,000 different substances. To be able to screen such amount of chemicals, a fast and reliable High Throughput assay is needed. The assay presented before are based on either SDS-PAGE gel electrophoreses or HPLC analysis. Both methods are not fast. The typical run of the HPLC assay was about 30 min. and for measuring kinetic enzyme reactions several data points are needed. We therefore designed a novel High Throughput assay for screening bacterial CTPs.
High Throughput assay development

A High Throughput assay has to meet several criteria. The assay has to be reliable, fast, sensitive, easy to measure with simple equipment, without much handling steps and to be cheap. Beebe and Pei developed a continues fluorimetric assay for Prc from E.coli (Beebe and Pei, 1998). They designed a synthetic peptide based on the tmRNA peptide from the same organism. The peptide was synthesized with a fluorescent donor/quencher couple. Therefore the fluorescent donor EDANS (5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid) was synthesized at the N-terminus of the peptide and the quencher DABSYL (4-(4-dimethylaminophenylazo)benzoic acid) was attached at a lysyl side chain leaving the scissile bound between the donor and quencher. The substrate is shown in figure 11.1A. The usage of a fluorescent donor/quencher couple substrate enables a fast and sensitive assay. The principle of the donor/quencher is based on the fact that a photon may excited the fluorescent donor group. After excitation the fluorescent group will emitted a photon of specific wavelength. This photon is being captured by the quencher group which transforms the energy into heat. The photon is therefore loss for detection. The quenching of the photon will only happen if the quenching group is in the approximation of the photon emitting group. If the quenching and fluorescent group is separated by hydrolysis of a covalent bound by cleavage the emitted light will not be quenched leaving the photon for detection. Cleavage activity can therefore be shown by the presence of emitting light of the fluorescent group.

Based on these principles we designed a novel substrate. The substrate had to be suitable for most bacterial CTPs. Therefore the substrate had to have a relativity broad specificity. The synthetic peptides of the plant species would meet this criteria. In order to be able to measure in a continue assay a fluorescent donor/quencher coupled (EDANS/DABSYL) was introduced in the pD1 peptide of S. obliquus. The resulting structure of the substrate is represented in figure 11.1B.
Figure 11.1: A) Prc *E.coli* fluorescent peptide substrate of Beebe and Pea. B) Chemical structure of the new designed fluorescent peptide substrate for bacterial CTPs.
Chapter 11

Modeling experiments were performed in order to check if the addition of the fluorescent donor/quencher groups to the substrate would fit within the active site of the protease. A picture of the modeling experiment in which the substrate is shown within the active site of the modeled CtpA of \textit{P. aeruginosa} is shown in figure 11.2. The modeling experiment showed that there is enough space in both the closed and the open conformation for the substrate and the EDANS group within the active site.

Based on these results cleavage experiments with the substrate were executed. Enzyme kinetics with the substrate were performed with CtpA from \textit{P. aeruginosa} and Prc form \textit{E.coli}. The results of three independent experiments with 9 different substrate concentrations is shown in figure 11.3. The enzyme kinetics were measured using a plate reader with a fluorescence spectrophotometer.

The CTP substrate is cleaved by both CtpA from \textit{P. aeruginosa} as by Prc of \textit{E.coli}. The cleavage of the substrate without the EDANS/DABSYL couple was already shown in Chapter 10. The CtpA showed enzyme kinetic parameters of $k_{\text{cat}}$ $0.057 \pm 0.003$ s$^{-1}$, $K_M$ $21.21 \pm 3.4$ μM and $k_{\text{cat}}/K_M$ $2.7 \times 10^3$ M$^{-1}$ s$^{-1}$. The Prc showed enzyme kinetic parameters of $k_{\text{cat}}$ $0.067 \pm 0.004$ s$^{-1}$, $K_M$ $17.94 \pm 2.7$ μM and $k_{\text{cat}}/K_M$ $3.7 \times 10^3$ M$^{-1}$ s$^{-1}$.

Beebe and Pea reported Michaelis-Menten constants of their substrate with the Prc of \textit{E.coli} of $k_{\text{cat}}$ of $0.086 \pm 0.002$ s$^{-1}$, $K_M$ of $4.0 \pm 0.3$ mM, and $k_{\text{cat}}/K_M$ of $2.2 \times 10^4$ M$^{-1}$ s$^{-1}$. Although the substrate is different from our CTP substrate the enzyme kinetics parameters are comparable.

The results of these cleavage experiments show that the designed CTP substrate is suitable for High Throughput screening purposes.
Figure 11.2: CTP Substrate within CtpA (modelled) of *P. aeruginosa*. A) In the closed conformation. B) In the proposed open conformation.
Figure 11.3: Michaelis-Menten enzyme kinetics of the CTP substrate. A) CtpA from *P.aeruginosa*. B) Prc from *E.coli*.
Inhibitor identification

As explained in chapter 10 CTPs of phototrophic organisms such as plants, algae and cyanobacteria play a role in the activation of the pD1. The mature D1 is a central protein within Photosystem II as part of the photosynthesis machinery. Together D1 and D2 unite as a heterodimer that forms the reaction core of Photosynthem II and facilitates electron transfer after photon absorption by the chromophores (Satoh and Yamamoto, 2007). Inactivity of the reaction centre abolishes photosynthesis and is therefore incompatible with life.

The fact that inactivity of CTPs are lethal for green organisms make these interesting targets for the development of novel inhibitors acting as herbicides. Duff et al. searched for novel lead structures as inhibitors of CtpA from Spinacia oleracea. This research was as collaboration between among other the companies Monsanto and Pfizer. Duff and co-workers screened approximately 500,000 chemical different compounds from various sources with a High Throughput screening assay. There identified several compounds and measured their IC₅₀ and Ki values. The compounds identified are presented in figure 11.4. The measured inhibitory parameters are shown in table 11.1.

The results show that a diverse class of compounds can inhibit CtpA from S.oleracea. Most compounds seems to inhibit competitive. Only the benzoxazinone was shown to be a uncompetitive inhibitor and irreversible. Based on the knowledge of the structure of CTPs one may suggest that the protease has to obvious sites for interaction with inhibitors namely the PDZ domain and the active site.

Based on these results we hypothesized that the compounds identified by Duff et al. would also have inhibitory activity against bacterial CTPs. After all the bacterial CTPs were able to process the pD1 peptide mimics of two different phototrophic organism. All compounds were tested in silico by performing docking studies on the modelled CtpA of P.aeruginosa (data not shown). These studies were preliminary but suggested that all the ketohetrocycles structures possibly bind within the active site and the CF₃ Peptides most likely bind to the PDZ domain.
Rhodanine dimer

Figure 11.4: Chemical structures of the compounds identified by Duff et al. as novel inhibitors of CtpA from *S.oleracea* (Duff et al., 2007).

Ketoheterocycles

Figure 11.4: Chemical structures of the compounds identified by Duff et al. as novel inhibitors of CtpA from *S.oleracea* (Duff et al., 2007).
Figure 11.4 continued: Chemical structures of the compounds identified by Duff et al. as novel inhibitors of CtpA from *S. oleracea* (Duff et al., 2007).
All compounds were checked for commercial availability. Only the rhodanine dimers could be purchased. Therefore Rhodanine dimer A and B were tested for inhibitory activity against CtpA from *P. aeruginosa* and Prc of *E. coli*.

The results of the inhibition measurement of both Rhodanine dimers A and B are shown in figures 11.5 and 11.6 respectively. Figure 11.5 shows the results of Rhodanine dimer A. Both CtpA and Prc are inhibited by the compound. Rhodanine dimer A had a IC$_{50}$ of 15.4 ± 1.5 μM and calculated $K_i$ of 9.0 ± 0.9 μM against CtpA from *P. aeruginosa*. For Prc from *E. coli* the determined IC$_{50}$ was 1.4 ± 0.08 μM and $K_i$ of 0.7 ± 0.04 μM. In order to compare this with the CtpA of *S. oleracea* the $K_i$ can be compared which is independent of the assay. Rhodanine dimer A has a $K_i$ of 3.8 μM with the CtpA of *S. oleracea*. These values are more or less comparable with the values found with CtpA and Prc.

The results for the inhibitory experiments for Rhodanine dimer B are shown in figure 11.6. The inhibitory parameters of Rhodanine dimer B for CtpA were IC$_{50}$ of 24.1 ± 1.0 μM and $K_i$ of 14.1 ± 0.5 μM. For Prc the following values were determined IC$_{50}$ of 8.9 ± 0.4 μM and $K_i$ of 4.9 ± 0.2 μM. Rhodadine dimer B showed a $K_i$ of 2.7 μM with CtpA of *S. oleracea*. These values are again comparable.

The results show that both Rhodanine dimers are inhibitors of the bacterial Carboxy-terminal processing proteases CtpA of *P. aeruginosa* and Prc of *E. coli*.
Table 11.1: Inhibitory parameters of tested compounds against CtpA from *S.oleracea*. identified by Duff et al. (Duff et al., 2007).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class</th>
<th>IC₅₀ (μM)</th>
<th>Ki (μM)</th>
<th>Mode of inhibition</th>
<th>Reversible</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Rhodanine dimer</td>
<td>8.0</td>
<td>3.8</td>
<td>Competitive</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>Rhodanine dimer</td>
<td>6.0</td>
<td>2.7</td>
<td>Competitive</td>
<td>Yes</td>
</tr>
<tr>
<td>C</td>
<td>Ketoheterocycles</td>
<td>50</td>
<td>20</td>
<td>Competitive</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>Ketoheterocycles</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>E</td>
<td>Ketoheterocycles</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>F</td>
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<td>Uncompetitive</td>
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</tr>
<tr>
<td>G</td>
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<td>45</td>
<td>Competitive</td>
<td>Yes</td>
</tr>
<tr>
<td>H</td>
<td>CF₃ Peptides</td>
<td>7.6</td>
<td>3.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>I</td>
<td>CF₃ Peptides</td>
<td>0.2</td>
<td>0.1</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>
Figure 11.5: Inhibition curves of Rhodanine dimer A with bacterial CTPs A) CtpA from P.aeruginosa B) Prc from E.coli.
CTP target inhibition: HTS assay and inhibitor screening

A
IC\textsubscript{50} 24.1 ± 1.0 μM
K\textsubscript{i} 14.1 ± 0.5 μM

Relative activity

Logarithmic conc. Rhodanine dimer B (M)

B
IC\textsubscript{50} 8.9 ± 0.4 μM
K\textsubscript{i} 4.9 ± 0.2 μM

Relative activity

Logarithmic conc. Rhodanine dimer B (M)

Figure 11.6: Inhibition curves of Rhodanine dimer B with bacterial CTPs A) CtpA from \textit{P.aeruginosa} B) Prc from \textit{E.coli}. 
Chapter 11

Chapter discussion

The development of a High Throughput assay is essential for screening large chemical libraries. The design of a substrate with a fluorescent donor/ quencher couple makes it possible to examine enzyme kinetics over times with one assay sample which is very sensitive. The designed substrate based on the D1 peptide mimic of S.oleracea with a EDANS/DABSYL couple showed to be suitable in an assay for both CTPs from two different bacterial species P.aeruginosa and E.coli. Because the substrate of a green organism was chosen with far evolutionary distance makes it plausible that the substrate may also be used with other CTPs from varying bacterial species.

The new substrate was used to identify novel inhibitors. The relation between CTPs from green organisms and the CTPs from P.aeruginosa and E.coli led to the assumption that also proven inhibitors may be effective for both species. The rhodanine dimmers A and B were shown to be able to inhibit both the CtpA of P.aeruginosa as well as Prc from E.coli.

This is the first time novel small molecular compounds were identified as inhibitors for bacterial CTPs. Rhodanine dimer A and B may therefore function as lead structure in the development as novel antibiotics. The finding of these inhibitors fulfill one of the last aims of this thesis, the discovery of new compounds that could act as novel antibiotics.

Future research has to confirm the inhibitory action in vivo and subsequent virulence attenuation has to be proven in infection models with P.aeruginosa and other bacterial species.
CTP target inhibition: HTS assay and inhibitor screening

References


"The system can be incapacitated in two ways; either by stopping the iteration or by disarming it"
CHAPTER 12

General Discussion
General Discussion

The ultimate goal for a pharmacologist is to find a new drug to cure a disease. In particular, the development of an anti-infective agent would be very rewarding. In history, agents that could cure infectious diseases are probably one of the most influencing drugs for mankind that have ever been developed. Since the development of antibiotics at the beginning of the 20th century the aim of these agents was to destroy the infection causing organism. Over the years, new antibiotics have been developed and helped to defeat these frequently occurring diseases. As the use of these antibiotics increased so did the development of resistance of the pathogen against these agents. To this date, antibiotics that have been developed and used as drugs are based on the destruction or growth inhibition of the pathogen.

The aim of the thesis was to find a novel target in *Pseudomonas aeruginosa* for the development of antibiotics. This target had to have the characteristic not to destroy the bacterium but to disarm the pathogen from its virulence. The hypothesis behind this idea is that resistance to these agents will be developed much slower than when an agent destroys the organism. The selection pressure would be much less.
During this research I tried to work according to the classical process of drug development. The difference stages within the classical process of drug development are:

**Target identification:**
identifying a biological target that can be modified so that it cures a disease.

**Target validation:**
Check if modifying the biological target indeed is able to cure a disease.

**Target isolation:**
isolation of the biological target for further development

**Target characterisation:**
characterisation of the biological target in order to understand its purpose and identify options to modify its action

**Target inhibition:**
identifying small molecular agents (lead compounds) that are able to modify the target in us a way that it cures a disease

After that a lead compound would be optimized for pharmacokinetics, effectiveness and toxicology. A resulting compound would be tested within clinical trials.
General Discussion

**The first stage was to identify a target**

There are two main principles necessary that make a bacterium pathogenic. First the bacterium has to be able to invade and evade the host defense system and secondly the pathogen has to have the ability to adhere and establish an infection. In order to fulfill these two principles a pathogen most often has to secrete virulence factors in its extracellular environment. This fact brought the focus on two related proteases in *P. aeruginosa*. The proteases belong to the novel serine protease family of Carboxy-terminal processing proteases. Mutant strains of *P. aeruginosa* with these proteases inactivated (ΔctpA and Δprc) showed reduced secretion of Lipase A. Besides that mutants strains of these proteases in other bacterial species showed a reduced virulence.

Based on these result I wanted to investigate the influence of these proteases on the secretion of other proteins especially virulence factors. These result are presented in Chapter 5. Proteomics (2D gel electrophoresis analysis) of the secretome was performed and several virulence factor were indentified such as Pseudolysin, Staphylolysin, Alkaline protease, Protease IV and the Aminopeptidase PepB that showed reduced secretion. These results were confirmed by the use of enzyme specific assays and showed that both CTP mutants are defective in the secretion of these virulence factors. Based on these experiments a possible mechanism could be postulated why other CTP defective mutants in different strains were less virulent. CTP are in *P. aeruginosa* clearly necessary for a functional secretion system. It is unlikely that only one secretion mechanism is effected by CTPs as the secretion of the identified virulence factors are mediated by different secretion mechanisms. Alkaline protease is secreted by a type I secretion system. Pseudolysin and Staphylolysin are secreted by a type II secretion system. The secretion pathways of Protease IV and the Aminopeptidase PepB are unknown until now. Therefore it seems plausible that CTPs influence secretion on a more basal level. The results of these experiments identified the CTPs CtpA and Prc of *P. aeruginosa* as two possible antibiotargets. These targets needed to be validated.

**The second stage was to validate the target**
In chapter 5 it was shown that the CTP defective *P. aeruginosa* mutants ΔctpA and Δprc were disrupted in the secretion of proteins among which major virulence factors. This led to the hypothesis that the ΔctpA and Δprc *P. aeruginosa* strains are less virulence. In order to test this hypothesis the CTP defective strains were evaluated in different infection models. Four different infection models were tested: Thale cress (*Arabidopsis thaliana*), Fruit fly (*Drosophila melanogaster*), *Caenorhabditis elegans* and the Greater Wax Moth (*Galleria Mellonella*). All models are well respected infection models for evaluating the pathogenesis of *Pseudomonas aeruginosa* (Jander et al., 2000).

Virulence of both ΔctpA and Δprc *P. aeruginosa* strains were attenuated in the *A. thalianana* model, the *D. melanogaster* feeding assay and the *C. elegans* slow killing assay. All of these models were non-invasive and depended greatly in over winning the hosts own defense mechanisms. There were no clear difference between the two CTP defective strains. These results suggest that the CTP gene products are important in establishing full virulence in the tested host organisms. The wide range genetic origin of the organisms show that the CTPs are involved in a general mechanism that influences pathogenesis of the bacterium.

In three other assays the virulence of both the ΔctpA and Δprc strains were tested in models with direct systemic infection. For these infections *P. aeruginosa* did not have to overcome the natural physical barrier present in these hosts. This was evaluated in the *D. melanogaster* needle pricking assay and the *G. mellonella* injection assay. In the needle pricking assay with *D. melanogaster*, virulence reduction was not that evident as seen with the feeding assay. There were both difference in survival prolongation for the ΔctpA and Δprc strain, which was significant for ΔctpA but not for Δprc. Overall killing of the systemic infected *D. melanogaster* is much faster than when fed on *P. aeruginosa*. *G. mellonella* was also infected systemically. In this assay both Δprc and the ΔctpA strain showed a significant difference in LD$_{50}$ values. In this case the Δprc was less virulent than the ΔctpA strain. Which is opposed from the observations in the *D. melanogaster* needle pricking assay.

As shown in Chapter 5 both CTP inactivated mutants are defective in the secretion of virulence proteases such as Pseudolysin and Staphylolysin, Protease VI, Alkaline protease and the Aminopeptidase PepB. Based on the results observed during the infection experiments these secretion defect influences the virulence of *P. aeruginosa*. The virulence protease could well
play a role in the destruction of psychical barriers in the host or other immune responses and by doing so establishing an entrance for systemic infection. Although other virulence factors can also be involved that haven't been identified in the secretome analysis.

But these virulence factors are not solely necessary for entering and establishing the infection as the other systemic infection experiments have shown. There was also a reduced virulence of the two CTP defective strain in these infection assays. Thus the virulence factors play also a role in the systemic infection of organisms. One experiment even showed a clear role for the secreted virulence factors of *P. aeruginosa* as sterile supernatant cultures were lethal for *G. mellonella*. This confirms that inactivating the CTP of *P. aeruginosa* leads to a decreased virulence by direct influencing the secretion of virulence factors.

In order to understand the mechanism behind the observed virulence attenuation further characterization of the target was necessarily.

In bacteria CTPs are involved in several basal physiological functions. There is some doubt about its real sub cellular localization. This information would indeed help in a better understanding of its physiological function and how this influences other cellular processes. In most literature CTPs from Gram-negative bacteria are referred to as periplasmic proteases. When these literature is examined in more detail the experimental evidence of this sub cellular localization is poor. There are two studies that form the fundamentals of this assertion. Both were performed on *E. coli* in the early 1990ies. Both experiments were carried out under rather non physiological conditions that could influences the outcome. Besides that both groups didn't take in account that the Prc could be secreted in the extracellular environment. This hypothesis would be interesting based on the results presented in chapter 5 and 6.

The subcellular localization of CtpA of *P. aeruginosa* was therefore further investigated by the use of fractionation experiments. As shown in Chapter 7 CtpA of *P. aeruginosa* is sub cellular localized in the periplasm. This observation rejects the hypothesis that CtpA could be secreted in the extracellular environment. The hypothesis was regenerated on the results of Lad and coworkers which showed that Tsp from *C. trachomatis* interfered with the NF-κB pathway (intracellular) by cleaving the p65 protein of the host in a human cell line. This could explain
how these obligate intracellular pathogens are able to evade the host own immune system. The most simple explanation would be that the Tsp of *C.trachomatis* facilitates this under the conditions that the protease is secreted in the extracellular environment or would be localized at least in the outer membrane of the pathogen.

This is clearly not the case for *P.aeruginosa* as the CtpA is solely localized in the periplasm. This gives additional information in the physiological function of CTPs in *P.aeruginosa*. As mentioned in Chapter 5 CTPs could be effector proteases that are secreted into the extracellular environment to regulate virulence factors. For example could the CTP protease be secreted outside and cleave virulence factors such as Pseudolysin, Staphylolysin or Protease IV and Alkaline protease and therefore activate them. Because CtpA is now shown to be localized in the periplasm this hypothesis is rejected. No direct effect of the protease can be expected in the extracellular environment, this effect has to be related to a periplasmic action of the protease.

The other CTP from *P.aeruginosa* Prc has not been sub cellular localized. Although it is plausible to assume that Prc is also localized in the periplasm.
The third stage was to isolate the target

For the elucidation of the physiological function of the protease and for the development of possible inhibitors it was necessary to clone and characterize these proteases. Therefore CtpA and Prc were cloned, expressed and purified successfully as shown in Chapter 8. The cloning and expression was difficult in several *E.coli* strains. It seems that the production strains experienced difficulties under certain expression conditions probably because the proteases interfered with physiological functions of the bacterium. This was illustrated by the fact that the only *E.coli* clone that overproduced CtpA contained a mutation within the approximation of the active residues. Which probably resulted in an inactive variant. These results confirm the hypothesis that CTPs are involved in basal cellular functions. Especially Prc from *P.aeruginosa* has a high resemblances to Prc from *E.coli* (38% identity and 50% similarity) which could be physiological active in this host.

Therefore non-viable expression system were tried such as *in vitro* transcription and translation systems in order to synthesis the desired proteases. Using these systems it was possible to synthesis the proteases CtpA and Prc and subsequent purification. Afterwards both proteases were successfully expressed in the *E.coli* BL21(DE3) under much milder conditions such as lower temperature and strict control of promoter leakage.

There was no evidence that the synthesized proteases were active. Activity of both CptA and Prc could be proven with a β-casein assay. CtpA and Prc both cleaved β-casein forming products of 20 and 15 kDa. The two products are probably subsequent cleavage products. This assay proofed that the expressed and purified proteases were active.

Further characterization was performed by mutagenesis of the proteases CptA and Prc. Putative active residues as predicted by multi-alignment analysis and indentified conserved amino acids within the catalytic domain. Alanine substitution mutation of the active residues serine 302 and lysine 327 in CtpA and serine 497 and lysine 504 were constructed, expressed and purified. These substituted variants of CtpA and Prc showed no activity in the β-casein assay. This indicates that both predicted active residues are indeed important for proteolytic activity in the cleavage of β-casein. The proposed mechanism that involves the serine and lysine forming a catalytic dyad maybe well valid for until now unknown physiological substrates.
The fourth stage was target characterization.

After the isolation it was necessary to further characterize the target in order to understand its physiological mechanism and be able to develop tools to find inhibitory compounds.

The physiological substrate of bacterial CTP proteases are yet unknown although there are some suggestions. It is suggested that Prc from E.coli cleaves Penicillin-Binding-Protein 3 (PBP3). PBPs are periplasmatic enzymes involved in the biosynthesis of the cell wall. E.coli PBP3 is thought to be a key element in cell septation in which it presumably initiates polymerization of the septum peptidoglycan by catalyzing a transpeptidation reaction during cell division (Nguyen-Distèche et al., 1998). Based on this, a new hypothesis could be introduced that CtpA and Prc of P.aeruginosa are involved in the processing of PBP3. This would be in evolutionary and functional equivalence to CTPs from phototrophic organisms. CTPs from Gram-negative bacteria may be required to activate periplasmatic proteins by cleavage as the photosynthetic D1 protein is activated in plant cells.

Genome mining of P.aeruginosa revealed two possible penicillin binding protein genes similar to PBP3 from E.coli. The first is named Penicillin Binding Protein 3 with an identity of 45% to PBP3 of E.coli and the second is Penicillin Binding Protein 3A with an identity of 42 %. The finding of two possible PBP3s matched the presence of two CTPs in P.aeruginosa which made the hypothesis stronger.

To test if the CTPs were able to process PBP3 and PBP3A, synthetic peptides were designed with a fluorescent label which enabled detection. The peptides represented the C-terminal part of the PBP3 and PBP3A protein with lengths of 38 amino acids and 29 amino acids respectively.

Previous observations were based on experiments with Prc of E.coli. Therefore the Prc from E.coli was also investigated within these experiments.

The results of these experiment were described in Chapter 9 showing that CtpA from P.aeruginosa was able to cleave PBP3 and PBP3A peptide substrates. Hereby CtpA seems to have an more preference towards the cleavage of PBP3 as shown by a higher relative activity. Prc was able only to process PBP3A. These results confirm the hypothesis that PBP3 can be processed by CTPs. It would make sense that if an organism has two distinct PBP3 proteins
that it has to have two proteases that can process the proteins. The results showed that there is no clear specificity towards one PBP3. Only Prc specifically processes the PBP3A and not the PBP3. But CtpA is able to cleave both protein. This difference in cleavage pattern may be involved in the complex mechanisms involved in the septation process. It seems more unlikely that the genome of *P. aeruginosa* would contain two proteases with exact the same function. If PBP3 and PBP3A are physiological substrates for CtpA and Prc needs to be investigated and confirmed by *in vivo* experiments.

Besides that the results show that Prc from *E. coli* can directly process the PBP3 from its own organism. This assay has never been executed before and they confirm early observations that Prc possibly cleavages PBP3. More strikingly Prc cleavages exact at the same site as was investigated in *prc* negative mutants. This makes the observations made about the *P. aeruginosa* proteases with the short synthetic peptides more valid. Besides that if PBP3 are confirmed to be physiological substrates of CTPs this would also explain their highly conserved presence in almost every bacterial genome known.

As PBP3 proteins are probably involved in cell wall biosynthesis and septum formation these cell processed could explain the earlier observation made in Chapters 5 and 6. A new hypothesis can be formulated that inactivation of the CTPs in *P. aeruginosa* disturbs or disrupts cell wall synthesis and septum formation. It is plausible that a malfunction in one of these processes could lead to a defect in the secretion of proteins and virulence factors. And this could lead to a disability in establishing an infection which was observed in the different infection models.

One can hypothesis that the formation of the septum is a crucial step during cell cyclus which would needed to be highly regulated and controlled. The CTPs could act as an activator of PBP3 to ensure an instant and onsite activation of the process. *De novo* synthesis of PBP-3 in a directly active form and subsequent translocation to the periplasm would probably be to slow or may lead to undesired side reactions within the cell.

Keiler et al. suggested CTPs could also be involved in the tmRNA tagging system. This system tags incorrectly synthesized protein in the ribosomes and directs them for degradation. This was also tested by using an artificial peptide. Both CtpA and Prc were able to cleavage this tmRNA specific peptide substrate. These results suggests that CTPs could be involved in
multiple cellular processes such as the tmRNA system. Again as with the PBP substrates these are artificial tmRNA peptides which mimic the C-terminal of proteins and the involvement of CtpA and Prc in this system has to be investigated in more detail.

The sequence of bacteria CTPs are highly similar to CTPs from phototropic organisms. Plants, algae and cyanobacteria have a CtpA protease encoded within their genomes. The CtpA plays its role in the activation of the D1 precursor protein which is part of Photosystem II.

It was therefore interesting to investigate whether bacterial CTPs are able to cleave the D1 precursor proteins from *S.oleracea* and *S.obliquus*. The cleavage ability of the bacterial CTP were there tested with synthetic peptides mimicking the C-terminal part of the D1 precursor protein. The results shown in Chapter 10 show that both CtpA and Prc as well as the Prc from *E.coli* were able to cleave the pD1 C-terminal peptide sequences. All CTP proteases cleaved the pD1 peptides substrates on the same sites as the native CtpA of these green organisms do. These results confirm that the bacterial CTP proteases are related to the CtpA proteases present in chlorophyll-b containing green organisms. The cleavage results together with the sequence similarity confirm their related origin. This result would also suggest a common physiological substrate but genome sequence searches did not reveal a possible candidate in *P.aeruginosa*.

In order to understand the mechanism involved in the hydrolysis of peptides with CTPs 3D modeling studies were executed. The modelled PDZ structure of CtpA from *S.obliquus* and that of *P.aeruginosa* showed both the same topology as described before by Liao et al. (Liao et al., 2000). A model experiment with the known C-terminal peptide of CtpA from *S.obliquus* was performed and revealed several key characteristics of the cleavage mechanism. First it seems that a proline residue at position P-4 is necessary to facilitate a turn which adapted the ligand to the CtpA surface and the Ser/Lys dyad active site. Secondly the model revealed a threonine Trh168 to be important for substrate recognition specificity as the residue interacts with the serine residue at P-3. Thirdly the PDZ seems to have two connecting loops which enables a hinge motion. This way the proteases could have different conformations, a closed or open form, which enables the protease to adjust itself depending on the length of the substrate. It is know that CtpA from *S.obliquus* can cleave at 9 and/or 16 amino acids from the C-terminus. Substrate binding on the PDZ domain and adjustment above the scissile bond within the active
site would not be feasible in the closed conformation. A hinge motion could explain the allowance of larger substrate peptide in the protease. The forth characteristic is a hydrophobic path in the vicinity of P1-P4 of the substrate peptide. The sequences at positions P1-P4 corresponds to “LDLA” which could interact with the hydrophobic path of the active site. This would suggest a second specificity characteristic of the substrate within the active site besides the C-terminus of the substrate.

Modeling of the CtpA from *P. aeruginosa* revealed evidence that the proposed mechanism of a catalytic dyad Ser/Lys is more plausible that a Ser/His/Asp canonical triad common in serine proteases.
The fifth stage was to identify novel inhibitors of the target

As mentioned in the introduction the ultimate goal for a pharmacologist is to find a new drug to cure a disease. This final stage was to identify novel inhibitors for bacterial Carboxy-terminal processing proteases. The function of the inhibitor should be to inactivate the CTPs and therefore attenuated the bacterium. The attenuated bacterium is then disarmed of his pathogenesis and therefore less infective.

Although I had shown several activity assays of the CTPs these weren’t suitable for inhibitor screening. In order to find novel inhibitors that may be used as lead compounds in the drug discovery process, small chemical compound libraries are screened with typical amounts of 10.000 to 1,000.000 different substances. To be able to screen such amount of chemicals, a fast and reliable High Throughput assay was needed. The assays presented before are based on either SDS-PAGE gel electrophoreses or HPLC analysis. Both methods are not fast. The typical run of the HPLC assay was about 30 min. and for measuring kinetic enzyme reactions several data points are needed. I therefore designed a novel High Throughput assay for screening bacterial CTPs. The design of a substrate with a fluorescent donor/ quencher couple makes is possible to examine enzyme kinetics over times with one assay sample which is very sensitive. The substrate designed based on the D1 peptide mimic of S.oleracea with a EDANS/DABSYL couple showed to be suitable as an assay for both CTPs from two different bacterial species P.aeruginosa and E.coli. Because the substrate of a green organism was chosen with far evolutionary distance makes is plausible that the substrate may also be used with other CTPs from varying bacterial species.

The new substrate was used to identify novel inhibitors. The relation between CTPs from green organisms and the CTPs from P.aeruginosa and E.coli led to the assumption that also proven inhibitors of the CTPs from green organism may be effective for both species. The rhodanine dimmers A and B were shown to be able to inhibit both the CtpA of P.aeruginosa as well as Prc from E.coli.

This is the first time that novel small molecular compounds were identified as inhibitors for bacterial CTPs. Rhodanine dimer A and B may therefore function as lead structure in the
development as novel antibiotics. The finding of these inhibitors fulfill one of the last aims of this thesis the discovery of new compounds that could act as novel antibiotics.

Future research has to confirm the inhibitory action in vivo and subsequent virulence attentions has to be proven in infection models with P. aeruginosa and other bacterial species.
Conclusion

During the research for this thesis I was able to indentify Carboxy-terminal processing proteases as novel targets for antibiotic therapy. The CTPs were validated in several infection models which showed reduces virulence. There is strong evidence that this virulence attenuation can be explained by the reduced secretion of virulence factors. CTPs from \textit{P.aeruginosa} were successfully cloned, expressed and purified which enable further research. I could show that the CTPs are involved in the cleavage of Penicillin-binding proteins 3 and 3A. But may also play a role in the tmRNA tagging system. Bacterial CTPs show high resemblances toward CTPs from phototrophic organism and are able to cleave precursor D1 proteins. The recombinant produced CtpA and Prc were active and the two putative actie site residues were showing to be important for activity. A High Throughput fluorescent/quencher peptide was designed and shown to be functional for screening purposes. Two rhodanine dimer compounds A and B were shown to be inhibitors for bacterial Carboxy-terminal processing proteases.
References


Addendum
Publications in relation to this thesis


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Rien
There is no greater thing you can do with your life and your work than follow your passions - in a way that serves the world and you...

Richard Branson