Novel applications of mass spectrometry-based proteomics in clinical microbiology

Fleurbaaij, F.

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“Success does not consist in never making blunders but
in never making the same one a second time.”

- Henry Wheeler Shaw, 1874

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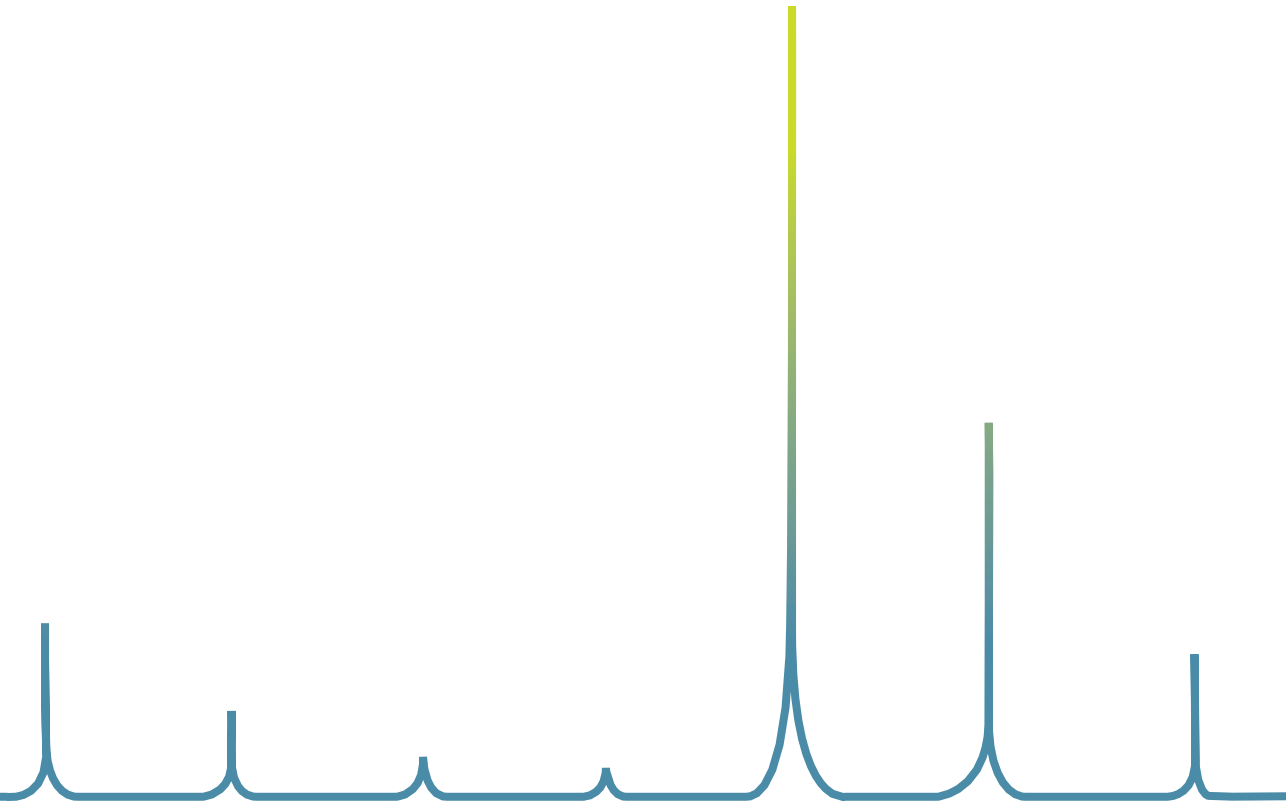
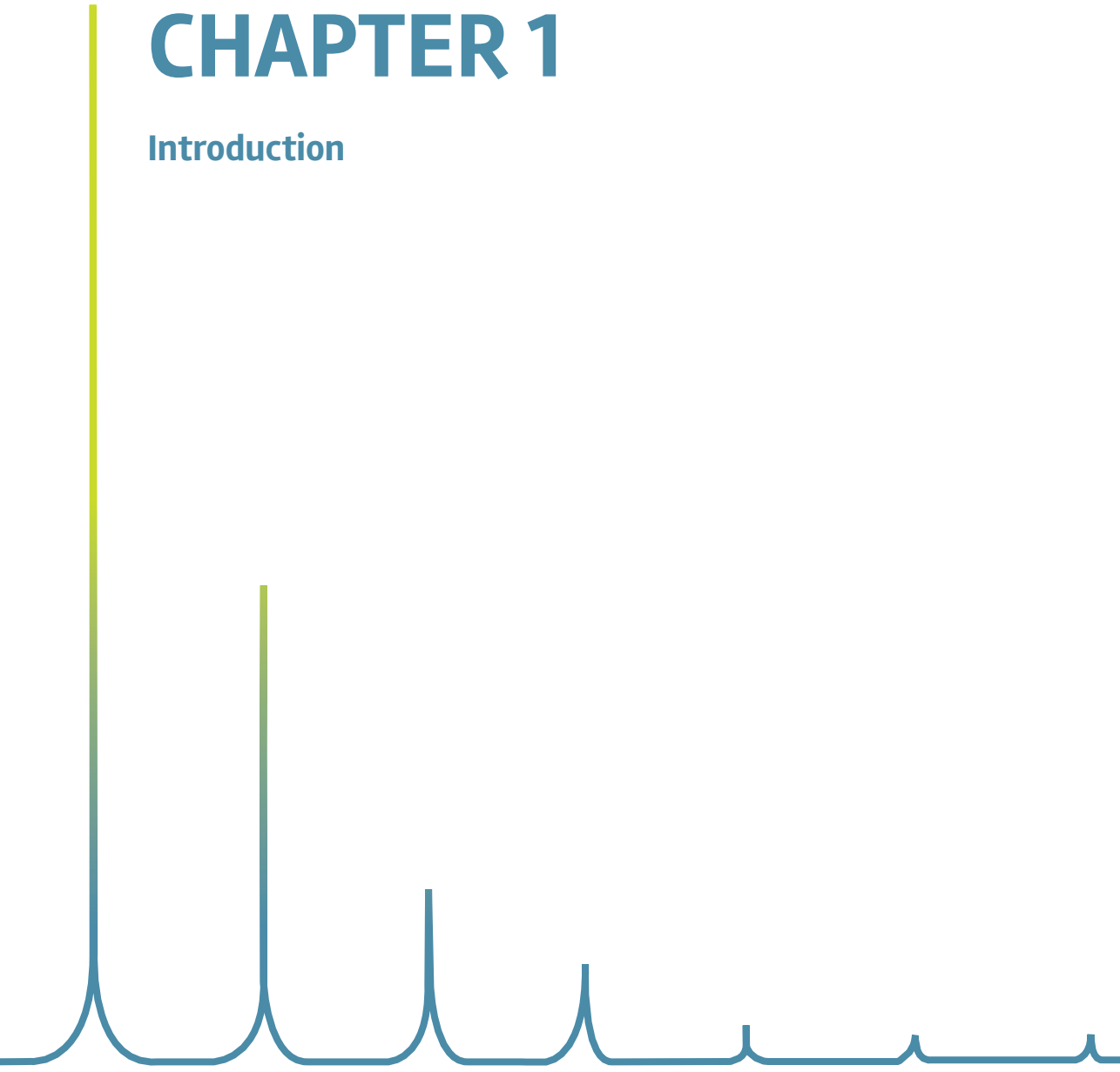


CHAPTER 1

Introduction



Gram–negative pathogenic bacteria

The domain of *Bacteria* is one of the three domains of life. According to the Manual of Clinical Microbiology[1], there are currently 34 formally named phyla (major lineages) as well as a large number of uncultured bacteria which are not yet classified. Taxonomy evolves alongside technology; previously, classification was based solely on morphological and biochemical traits but nowadays genotypic data (such as 16S RNA and recently more advanced genomic data) are used for these classifications. Using traditional high-order taxonomy (based on rRNA sequences), the majority of clinically relevant species are found in three phyla[1]. These are the *Firmicutes* and *Actinobacteria*, consisting of Gram-positive bacteria with low and high GC-content (the percentage of guanine and cytosine in the genome) respectively. The third phylum, *Proteobacteria* is one of the largest phyla and comprises many important human pathogens. The phylum can be divided into five different classes from *Alphaproteobacteria* to *Epsilonproteobacteria* and contains the majority of known Gram-negative bacteria. New classes are still being proposed, such as the *Zetaproteobacteria*[2] and the *Acidithiobacillales*[3]. Each of these groups contains a number of important families. One of these is the Enterobacteriaceae, which are part of the *Gammaprotobacteria*. The Enterobacteriaceae are a large group of rod shaped Gram-negative bacteria. Enterobacteriaceae are facultative anaerobes, which means that in the presence of oxygen they use aerobic respiration to create ATP for energy and growth, but can switch to (glucose) fermentation if oxygen is not present. They are typically oxidase negative and non-spore forming[1]. The numbers vary, depending on taxonomical definitions but around 50 genera and more than 200 species are recognised[4]. These include many clinically important species like *Salmonella enterica, Escherichia coli, Klebsiella pneumoniae, Citrobacter freundii, Enterobacter cloacae, Proteus mirabilis* and more. Outside of the Enterobacteriaceae there are still more clinically relevant Gram-negative pathogens, such as *Pseudomonas aeruginosa*, part of the *Gammaproteobacteria*, which does not belong to the Enterobacteriaceae, as it is a non-glucose fermenter in the absence of oxygen. *P. aeruginosa* is an opportunistic pathogen which is an important agent of hospital acquired infections.

Bacterial identification: the MALDI–TOF MS revolution

Traditionally, identification of bacteria is based on phenotypic characteristics using a combination of Gram-staining, culturing and biochemical methods[5]. In recent years, mass spectrometry (MS) and more specifically, matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry has emerged as a platform that has revolutionised clinical microbiological practice[6]. Following bacterial culture, cells are applied either directly or after an extraction to the MALDI target plate. The extracts are typically acidified with formic acid to aid the ionisation process and a matrix is applied on top. The samples are then dried, during which a complex of matrix and analytes is formed. During analysis, laser shots are fired at the dried spots, where the matrix absorbs the majority of the energy, which

prevents extensive analyte fragmentation by the laser. The matrix and analyte complex is desorbed and ionized due to this energy, with the matrix typically being ionized by the laser and then transferring its charge to analyte ions in the gas phase. In MALDI-TOF MS analysis of bacteria, it is primarily the proteins that are brought into the gas phase and ionized. These gas phase ions can then be directed into the mass spectrometer and analysed. This results in protein spectra, where the majority of the peaks represent the major protein species, namely ribosomal proteins[7]. Despite this limited complexity, the profile generated in this analysis (*m/z* coordinate versus intensity) is discriminating enough to reliably generate a species level identification. This is achieved by comparing the generated spectra to a library containing reference spectra of a large collection of clinically relevant species resulting in a similarity score. With the ever increasing amount of fully sequenced bacteria, using bioinformatics approaches to compare spectra to predicted spectra based on whole genome sequencing data is generating interest as well[8]. Currently the library based approaches are more common, but this could potentially change in the future with the advent of more sequencing data and a reduction in cost of bioinformatics based approaches[8]. Whichever method is used, the application of MALDI-TOF MS is very rapid (with analysis times of <30 mins) compared to its diagnostic peers[9]. Combined with the sensitivity and accuracy of the platform[10], this has resulted in the widespread adoption of MALDI-TOF mass spectrometry for microorganism identification and these platforms have had a significant impact on diagnostics and treatment of infections[11]. In clinical microbiology, the introduction of MALDI-TOF MS has resulted in faster diagnostics, higher throughput and cost reduction[6]. This is associated with the availability of standardized, validated and approved platforms like the Bruker Biotyper (Bruker Daltonics, Bremen, Germany) and Vitek MS (bioMérieux, Marcy-l'Étoile, France). On top of this, MALDI-TOF MS is positioned to take advantage of further automation efforts in microbiological practice, which requires high-throughput analysis[12]. The broad applicability of the technique makes that these benefits are applicable for all microbiological diagnostics, whether the species are of clinical, veterinary or environmental origin[13-15].

Bacterial typing for Gram–negative bacteria.

Bacterial typing, according to the recommendation established by Van Belkum[5] is defined as follows:

"Phenotypic and/or genetic analysis of bacterial isolates, below the species/subspecies level, performed in order to generate strain/clone-specific fingerprints or datasets that can be used, for example, to detect or rule out cross-infections, elucidate bacterial transmission patterns and find reservoirs or sources of infection in humans. 'Subtyping', a term commonly seen in American literature, is often used as a synonym for typing".

Hence, bacterial typing refers to the process of distinguishing bacteria on a level below species. This leads to the term bacterial strain, which according to Van Belkum, are all isolates that are descendent of a single isolation[5]. While identification to the species level is important, there is a need for more rapid methods to characterise and type individual isolates. In most studies, typing is used to assess (local) outbreaks or in broader (retrospective) epidemiological studies[16]. Moreover, successful differentiation between strains can also provide phenotypical insight[17] when a certain (set of) marker(s) is associated with e.g. pathogenicity, virulence or antibiotic resistance.

In general, molecular approaches are well established for bacterial typing and many different DNA fingerprinting methods have been developed. Table 1 is adapted from the 2015 Manual of Clinical Microbiology and illustrates a number of these methods[1]. Pulsed field gel-electrophoresis (PFGE) is used on microbial DNA, cut with specific enzymes and analysed by gel electrophoresis. The resulting band pattern is a unique fingerprint. PFGE has a long history (20-30 years) in microbiology and has been applied for practically all bacteria[18]. Due to its universal applicability, near 100% typeability (i.e. in any given collection nearly all strains can be typed), high discriminatory power, good reproducibility and stability[1]. The downsides are mainly limited to the requirement for specific electrophoresis equipment and the laborious nature of the analysis, where the typing of 20 isolates takes roughly two working days when performed by a single person[1]. Another technique is amplified fragment length polymorphism (AFLP). In AFLP analysis, a DNA fingerprint is generated in three steps[19]. First, the DNA is cut into smaller pieces using one or two restriction enzymes and the resulting fragments are ligated with oligonucleotide adapters. Next, a subset of the fragments is amplified by PCR. The amplified fragments are then separated and visualised either on polyacrylamide gels or by capillary electrophoresis with fluorescence detection. It can be used in case of outbreaks to investigate strain similarity. While perhaps not as widely applied, it is reported as a typing method for various species including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*[20-22]. Typically, it has a good intra-laboratory reproducibility, but exchange between laboratories is limited, even when appropriate standardization efforts are made[1]. Multiple-locus variable number of tandem repeat analysis (MLVA) is another DNA fingerprinting analysis. Here discrimination is based on the number of repeats in genes and intergenic regions of DNA. MLVA is especially valuable in combination with other typing methods such as PFGE, to specifically discriminate the clonal variants. Examples include extended-spectrum beta-lactamase (ESBL) producing E. coli and A. baumannii[23,24]. Multi-locus sequence typing (MLST) is based on sequencing techniques. It assesses the sequences (350 – 600 bp in length) of a number (5-10) of housekeeping genes[25]. MLST data can be readily compared between laboratories, which has resulted in curated databases (http://pubmlst.org, http://www.mlst.net, http://www.pasteur.fr/mlst) for pathogens such as E. coli, S. enterica and other Gram-negatives. The technique has high reproducibility and high typeability. However, it is not widely applied, due to the labour intensity and cost of

Table 1: Characteristics and application of a number of subtyping methods. Adapted from the Manual of Clinical Microbiology 2015.

Characteristic	PFGE	Whole-genome mapping	PCR-ribotyping	AFLP	MLST	MLVA	Whole-genome sequencing	Mass spectrometry*
Reproducibility	Good	Good	Good	Good	Good	Good	Good	Good
Stability	Good	Good	Good	Good	Moderate to good	Moderate to Good	Moderate to good	Good
Discriminatory Power	Excellent	Excellent	Good	Excellent	Low to moderate	Excellent	Excellent	Poor
Universal applicability	Yes	Yes	Yes	Yes	No	No	Yes	Yes
Applicability for library subtyping	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Complexity of data	Complex	Complex	Simple	Complex	Simple	Simple	Very complex	Simple
Ease of use	Moderately labor-intensive	Moderately labor-intensive	Simple	Moderate	Simple to moderately labor-intensive	Simple	Labor-intensive	Simple
Cost	Moderately labor-intensive	High	Low	Moderate	Moderate	Moderate	High	Low
Suggested use of method	Outbreak surveillance, large-scale libraries	To aid in the assembly of next-generation short sequence reads	First line subtyping of *C. difficile*	Local outbreak surveillance, suitable for local library subtyping	Phylogenetic studies, attribution of *Campylobacter*, potential forensic use	Outbreak surveillance, large-scale library subtyping if standardized, potentially good for forensic and attribution purposes	Outbreak investigation, (large-scale) library subtyping, phylogenetic studies, forensic microbiology, attribution	Subspecies level (serotype, pathotype) typing

*based on MALDI-TOF MS methodologies only.

sequencing DNA in both directions in multiple loci. Finally, whole genome sequencing (WGS) is increasingly applied for bacterial typing. In whole-genome typing every generated sequence read is compared directly to a reference genome. Thus, the quality of the analysis is directly related to the quality of the reference. With more and more sequencing data becoming available, finding a proper reference is becoming increasingly straightforward. Using WGS entire genomes of organisms can be analysed and compared directly and completely[26]. Resulting data can be submitted to online repositories such as the Bacterial Isolate Genome Sequence Database (BIGSdb, https://pubmlst.org/software/database/bigsdb/), to be able to compare to reference genomes and provide reference for future experiments. WGS has been applied in the analysis of many bacterial Gram-negative species, including *E. coli*[27-29], *K. pneumoniae*[30-32] and *P. aeruginosa*[33-35]. Naturally, this kind of approach and the data it yields is very complex and expensive to perform[36]. With time, the costs of sequencing has gone down and is expected to further drop in the near future. Similarly, WGS analysis presents a great bioinformatics challenge and further improvements in analysis methods and computing will have a beneficial effect on the wider applicability of WGS.

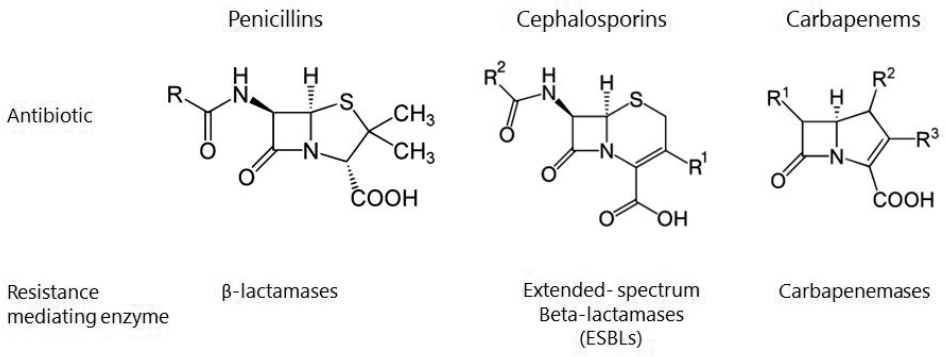
Due to the success of MALDI-TOF MS for species identification, attempts have been made to apply the technique for typing of bacteria. Reports for many bacteria have been published and appear promising. This includes species such as *E. coli*[37,38], *K. pneumoniae*[39] and other Gram-negatives. However, when trying to replicate these studies or perform them on a larger scale or in multiple laboratories, the limitations of this approach become apparent[40,41]. MALDI-TOF MS typing is not robust enough and the technique does not possess sufficient discriminatory power to overcome differences in databases, laboratories, culturing, extraction methods, matrices and other factors that influence the results of analysis. As it stands, MALDI-TOF MS based typing will not revolutionise the field in the way it did achieve for species level identifications. An interesting alternative is utilising MALDI ionisation with ultrahigh resolution mass spectrometry. This maintains the speed advantage of MALDI, while exceeding the resolution power of linear time-of-flight analysis, so that more in-depth characterisation of bacterial extracts should be possible. MALDI Fourier transform ion cyclotron resonance (FTICR) can provide such high resolution spectra. In FTICR analysis, ions are analysed in a fixed magnetic field, where the frequency at which they cycle around the magnet (the cyclotron resonance) is dependent on their mass to charge (*m/z*) ratio. The ions move in packages of similar *m/z* ratio and each time they pass an electrode they induce a charge. The frequency at which this happens is measured and can be translated into a very accurate mass to charge ratio. Therefore, MALDI-FTICR MS can isotopically resolve ion signals where MALDI-TOF MS cannot. MALDI-FTICR has been applied for the analysis of proteins in human serum, demonstrating its capabilities in complex matrices[42-44].

Antibiotics and resistance

Beta lactams

Since the early discovery of penicillin by Alexander Flemming in 1928, beta-lactams are still the most widely administered class of antibiotics in hospitals. They work by inhibiting the activity of transpeptidases that are involved in the assembly of the bacterial cell wall[45]. Bacterial resistance mechanisms to ß-lactam antibiotics are primarily based on the synthesis of ß-lactamases which are capable of degrading ß-lactams. Other mechanisms involve reduced uptake, due to decreased outer membrane permeability, or active efflux. Figure 1 depicts an overview of the three groups of beta-lactamases. The beta-lactamases can be classified by Amber class, which groups beta-lactamase enzymes by amino acid structure[46]. These range from A to D and provide an evolutionary perspective on the origin of the various beta-lactamases. However, such classification is not informative with regard to the function of the beta-lactamases. A functional classification scheme of beta-lactamases exists as well and the recognised system was first proposed in 1995 by Bush et al.[47] and updated in 2010[48]. It recognises substrates and inhibitor profiles and there is correlation with the structural classification in the major groups. Table 2 contains this functional classification[48].

Figure 1: Beta–lactam based antibiotics and their associated beta–lactamases.



Following the initial introduction of penicillin, new classes of beta-lactams have been developed, and currently cephalosporins and carbapenems form the major groups. Cephalosporins are originally derived from the fungus *Cephalosporium*, and each new generation of chemically modified cephalosporins show better (extended-spectrum) activity against Gram-negatives. The third generation cephalosporins such as ceftazidime and cefotaxime are widely used as antibiotic agents with a broad activity[49]. They feature an oxyimino side-chain, which hinders the binding of penicillinases[50]. They are used to treat infections with Enterobacteriaceae like *E. coli* and *K. pneumoniae* but ceftazidime also has activity against *P. aeruginosa*[51]. Enzyme modification of cephalosporins is mediated by a number of beta-lactamases, with AmpC (class 1, table 2) and extended-spectrum

Table 2: Classification schemes for bacterial beta–lactamases. Adapted from Bush, Jacoby, Updated functional classification of beta–lactamases, 2010.

Bush-Jacoby group	Molecular class	Substrates	Inhibited by		Characteristics	Representative enzymes
			CA/TZB	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	E. coli AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-- lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	PER-1, VEB-1, TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino- lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Cabenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino--lactams	OXA-11, OXA-15
2df	A	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino--lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CerA, IND-1
	B (B3)					
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	Cpha, Sfh-1

beta-lactamases (ESBLs, class 2be, table 2) being the most widespread[52]. AmpC beta-lactamases are chromosomal in a number of Enterobacteriaceae, but can also be plasmid-mediated resulting in dissemination among many Gram-negatives[53].

Similarly, the ESBLs can also be acquired through plasmids. There are three recognised types of ESBL (TEM, SHV and CTX-M)[54]. The TEM and SHV ESBLs are variants of previously known class A beta-lactamases, with a mutation in the active site that allows the conversion of beta-lactams with an oxyimino side-chain, antibiotics previously resistant to beta-lactamases[54]. The third type, CTX-M, are all ESBL. There are five subgroups recognised (1, 2, 8, 9 and 25, named after their structural archetype)[55,56]. First described in the late 1980s, the CTX-M ESBLs have spread rapidly and globally in the past 15 years and have become the most prevalent ESBLs[57]. Resistance to third generation cephalosporins is frequently observed combined with aminoglycoside and fluoroquinolone resistance[58]. This is due to the colocalization of resistant genes on plasmids, transmitting ESBLs[59]. Treatment options in case of multidrug resistance are usually limited to antibiotics such as carbapenems[60].

The carbapenems are the third class of medical important beta-lactam antibiotics. They have the broadest range of activity and are typically used as a last resort treatment in case of infections caused by bacteria resistant to other antibiotics . As with all beta-lactam antibiotics, the main path to resistance is through enzyme mediated modification of the carbapenems[57]. However, loss of number or activity of bacterial cell wall porins is also known to result in carbapenem resistance[61]. The outer membrane porin families OmpF and OmpC are instrumental for antibiotic uptake in Enterobacteriaceae. A reduced porin function combined with such beta-lactamases as AmpC and ESBLs can result in carbapenem resistance[62-64]. However, the main mediators of resistance are the carbapenemases[65]. A number of different types of carbapenemases exist, such as KPC, OXA-48, IMP, VIM and NDM. They derive from enzymes with differing amino acid sequences. Clinically, *Klebsiella pneumoniae* Carbapenemase (KPC) is the most important representative of class A carbapenemases, due to its spread and activity. Class B consists of metallo-beta-lactamases (MBLs) with carbapenemase activity. Resistance to this type of carbapenemase may vary[65]. The emergence of NDM-1 (named after New Delhi where it was initially identified) in the past decade is especially worrying, as it is found in many different species, appears to be readily acquired by *K. pneumoniae* and *E. coli* and has spread significantly, with especially India being considered an environmental reservoir[65,66]. Of the class D beta-lactamases, OXA-48 is the most frequently found type. Initially identified in Turkey, OXA-48 has spread significantly including numerous outbreaks in Europe[67,68]. OXA-48 is very difficult to detect, due to its low activity[69]. This results in unrecognized hospital outbreaks which present a high risk, especially in vulnerable patients[70].

Aminoglycosides

The aminoglycosides are bactericidal agents that bind to proteins of the 30S subunit of bacterial ribosomes, thereby interfering with protein synthesis[71]. Aminoglycosides are useful as a therapeutic agent against most aerobic Gram-negative bacteria but their toxicity is an important disadvantage[72,73]. However, due to the increased prevalence of antibiotic resistance of other drugs, they are considered as an important backup in case of multidrug resistance. Resistance to aminoglycosides is therefore a worrying prospect, especially when it occurs in bacteria already harbouring ESBL and carbapenem resistance[73]. There are multiple ways to mediate aminoglycoside resistance. A reduced uptake by limiting membrane permeability and an increasing efflux by pumps may result in resistance[71]. The major resistance mechanism is by the expression of aminoglycoside modifying enzymes. These enzymes contain three major classes with the aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs)[72]. These modifications reduce the binding affinity of the aminoglycosides, which results in a loss of activity. Modification of the target of aminoglycosides also result in resistance. Ribosomal mutations can occur and result in aminoglycoside resistance[73]. Additionally, methylation of the aminoglycoside binding sites by 16S ribosomal RNA methyltransferases (MTases) also results in resistance to aminoglycosides[73]. Plasmid mediated MTases have spread increasingly since the 2000s, and limit the use of aminoglycosides[74].

Polymyxins

Polymyxins are antibiotics that target the outer cell membrane of Gram-negative bacteria[75]. They destabilize the lipopolysaccharide (LPS) which results in increased membrane permeability and cell death[76]. As with the aminoglycosides, the use of colistin (polymyxin E), the major polymyxin antibiotic, is limited due to its nephrotoxic effects[75]. In the past decade, its therapeutic use has increased against multidrug resistant (MDR) organisms. It is mostly reserved as a last resort antibiotic in MDR bacteria that are also not susceptible to carbapenems[75]. Colistin resistance is therefore a major threat. A few bacterial species have intrinsic polymyxin resistance, by modifying the LPS which results in an increased electrostatic charge which prevents leaking[77,78]. Acquired resistance to polymyxins is of more concern, such as the emergence of the colistin resistance (mcr-1) containing plasmid, which confers resistance towards polymixins that was first reported in late 2015[79,80]. The sudden emergence of mcr-1 illustrates how new threats can develop and change the landscape overnight, further emphasising the importance of better diagnostic methods of resistance detection. In 2016, a mcr-2 mediated resistance has also been identified[81]. It shares a 77% nucleotide similarity with mcr-1 and appears to have spread more readily in colistin resistant bacteria of veterinary origin, most likely owing to the IncX4 plasmid that carries mcr-2[81]. A third plasmid, mcr-3 has been described as well[82]. Currently, six variants of the mcr-1 gene have been described in human and animal isolates of *E. coli*, *K. pneumoniae* and *Salmonella enterica serovar Typhimurium*. Moreover, other forms of transferable plasmid-mediated

colistin resistance genes related to mcr-1 have also been found amongst Enterobacteracaeae and Aeromonas spp. with a worldwide dissemination in humans and animals[75,83]. Simultaneously the emergence of plasmid-mediated colistin resistance also highlights the impact of globalization in terms of travel and transport of meat products on the spread of microorganisms, as well as the effectiveness of surveillance programs in making these trends visible[84].

Table 3: List of the top 18 antibiotic resistant threats according to the Centers for Disease Control and Prevention (CDC).

CDC	
Urgent threats[1]	
	Clostridium difficile
	Carbapenem-resistant Enterobacteriaceae
	Neisseria gonorrhea
Serious threats[2]	
	Multidrug-resistant *Acinetobacter*
	Drug-resistant *Campylobacter*
	Fluconazole-resistant Candida
	Extended spectrum beta-lactamase producing Enterobacteriaceae (ESBL)
	Vancomycin-resistant *Enterococcus*
	*M*ultidrug-resistant *Pseudomonas Aeruginosa*
	Drug-resistant *Salmonella* Serotype Typhi
	Drug-resistant *Shigella*
	Methicillin-resistant *Staphylococcus aureus*
	Drug-resistant *Streptococcus Pneumoniae*
	Drug-resistant Tubercolosis
Concerning Threats[3]	
	Vancomycin-resistant *Staphylococcus Aureus*
	Erythromycin-resisant Group A *Streptococcus*
	Erythromycin-resistant Group B *Streptococcus*

1: High-consequence antibiotic-resistant threats, identified across several criteria. These threats may not be currently widespread but have the potential to become so and require urgent attention to identify infections and limit transmission.

2: Significant antibiotic-resistant threats. Not considered urgent currently, but threats will worsen and become urgent without proper health monitoring and prevention activities.

3: Concerting. Current threat level for these is low and/or there are multiple therapeutic options for resistant infections. Threats in this category require monitoring and in some cases rapid incident or outbreak response.

Prevalence of antibiotic resistance bacteria

In general, infections caused by antibiotic resistant Gram-negative bacteria are an increasing problem in hospitals worldwide. The organisms are widespread and pose a serious threat, as they are associated with high morbidity and mortality rates[85].

Various institutions like the World Health Organisation (WHO) and the Centers for Disease Control and Prevention (CDC) keep track of the most urgent threats due to antibiotic-resistant organisms. Table 3 shows the current top threats according to the CDC. In a 2014 report, the WHO specifically highlights nine common pathogens as bacteria of international concern, due to their widespread nature and resistance towards commonly used antibiotics[86]. In Europe, the European Centre for Disease Prevention and Control (ECDC) estimates that infections caused by resistant bacteria are responsible for roughly 25,000 deaths in Europe annually, as noted in the Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2015[60]. Simultaneously, the cost of avoidable deaths, healthcare and productivity loss was estimated to be around EUR 1.5 billion[87]. Infection due to Gram-negative organisms are a particular concern, with reports of increasing resistance to third generation cephalosporins, often combined with fluoroquinolone and aminoglycoside resistance[88]. In the Netherlands, the number of reported multidrug-resistant infections has been stable in the period between 2011 and 2015[89]. In this 5 year period, 300000 isolates from 23 laboratories were evaluated. Highly resistant microorganisms (HRMO) were found amongst *E. coli* (8%) and *K. pneumoniae*(8-10%). In the Dutch guidelines, HRMO are defined as follows[90]:

"HRMO are defined as microorganisms which 1) are known to cause disease, 2) have acquired an antimicrobial resistance pattern that hampers (empirical) therapy, and 3) have the potential to spread if – in addition to standard precautions – no transmission-based precautions are taken."

Carbapenem resistance is still relatively rare in the Netherlands, with incidence reports of 0.01% and 0.19% for *E. coli* (n=148081) and *K. pneumoniae* (n=22626) respectively[89]. Table 4 is derived from NethMap report and shows the use of antibiotics for systemic use in hospitals over 10 years in Defined Daily Doses (DDD), illustrating the increasing use of antibiotics[89]. This increase is largely due to the increased use of cephalosporins and carbapenems. Table 5 gives an overview of the major classes of antibiotics for therapeutic use, their mechanism of action and mechanisms of resistance.

Table 4: Ten years use of antibiotics for systemic use (J01) in hospitals. 2007–2016 (Source: SWAB). expressed in DDD/100 patient–days.

Therapeutic Group	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Tetracyclines	2.57	2.66	2.67	2.67	2.60	2.49	2.33	2.23	2.25	2.11
Penicillins with extended spectrum	1.91	1.91	1.89	1.81	1.91	1.94	1.99	1.94	2.13	2.09
Beta-lactamase sensitive penicillins	0.46	0.42	0.39	0.37	0.35	0.33	0.31	0.30	0.23	0.24
Beta-lactamase resistant penicillins	0.32	0.36	0.38	0.38	0.39	0.41	0.41	0.44	0.43	0.45
Penicillins +becta-lactamase inhibitors	1.66	1.71	1.74	1.80	1.82	1.82	1.67	1.55	1.56	1.51
Cephalosporins	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03
Trimethoprim and derivatives	0.22	0.21	0.21	0.20	0.20	0.19	0.17	0.16	0.14	0.14
Suphonamides + trimethoprim	0.36	0.36	0.35	0.35	0.34	0.33	0.29	0.28	0.28	0.28
Macrolides	1.39	1.36	1.33	1.31	1.34	1.34	1.22	1.18	1.20	1.17
Lincosamides	0.10	0.11	0.12	0.14	0.15	0.16	0.17	0.18	0.19	0.20
Aminoglycosides	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.00
Fluoroquinolones	0.91	0.89	0.86	0.85	0.82	0.80	0.76	0.79	0.77	0.74
Nitrofuran derivatives	1.07	1.13	1.17	1.23	1.31	1.38	1.37	1.40	1.40	1.40
Methenamine	0.03	0.02	0.03	0.04	0.03	0.04	0.03	0.03	0.02	0.01
Antibiotics for systemic use (total)	**11.10**	**11.24**	**11.21**	**11.23**	**11.37**	**11.34**	**10.80**	**10.53**	**10.67**	**10.39**

Table 5: List of major antibiotics for clinical use and the major resistance mechanisms.

Class	Subclass	Examples	Mechanism of action
Beta-lactam			Beta-lactams bind to penicillin-binding-proteins rather than d-alanyl-d-alanine. This prevents crosslinking between the peptidoglycan layers from occurring, resulting in cell wall synthesis inhibition
	Penicillins	Benzylpenicillin, Amoxicillin	
	First generation cephalosporins	Cefalotin, Cefadroxil,	
	Second generation cephalopsorins	Cefprozil, Cefuroxime	
	Third generation cephalosporins	Cefotaxime, Ceftazidime	
	Carbapenems	Meropenem, Imipenem, Ertapenem	
Quinolones	Fluoroquinolones	Ciprofloxacin, Levofloxacin	Inhibition of the topoisomerase ligase domain, resulting in DNA fragmentation
Aminoglycosides		Amikacin, Gentamicin Tobramycin, Kanamycin	Binds 16S rRNA, thereby preventing elongation of nascent chain and disturbs proofreading, resulting in protein defects
Glycopeptides		Vancomycin, Teicoplanin	Complexes with the D-alanyl-D-alanine portion of peptide precursor units. This prevents cross-linking of the peptidoglycan and inhibits cell wall synthesis
Tetracyclines		Tetracyline, Tigacycline	Binds to bacterial ribosomes and inhibits amino acyl-tRNA, preventing peptide synthesis
Macrolides		Azithromycin, Clarithromycin	Macrolides bind to the 50s subunit of bacterial ribosomes, interfering with protein syntheis
Cotrimoxazole			Antibiotic consisting of trimethoprim and sulfamethoxazole. Both interfere with folic acid synthesis, which is a requisite for DNA synthesis
Resistance mechanisms			
Enzymatic inactivation or modification of antibiotic		Beta-lactamases, aminoglycoside modifying enzymes	Binding of antibiotic before it finds real target or modify structure to no longer function
Target modification		Modified Penicillin-binding protein, plasmid-mediated quinolone resistance	Replacement or modification of antibiotic targets to limit or remove their effect
Reducing antimicrobial accumulation		Modified porin expression, efflux pump	Decreasing the level of antibiotic present by either limiting uptake or increasing discharge

Detection of antibiotic resistance

In case of a (suspected) infection, rapid identification and availability of antibiotic susceptibility patterns of the bacterium are important for patient treatment. To properly define antibiotic resistance, a number of parameters are needed. As stated in the Manual of Clinical Microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a substrate at which no visible growth of the tested microorganism is observed[1]. If susceptibility testing for an antibiotic is evaluated for a bacterial species, the terms MIC50 and MIC90 are used. These are the concentrations that inhibit growth in 50% (MIC50) or 90% (MIC90) of the isolates. National and international guidelines have been developed for standardization of antibiotic susceptibility tests and its interpretation. Two large organisations, the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) provide these guidelines. According to the EUCAST criteria[91], two different interpretations are provided, namely a clinical breakpoint and an epidemiological cut-off value. A bacterium is clinically susceptible (S) when the micro-organism is susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success. Clinically resistant (R) micro-organisms are resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure. Clinical breakpoints are dependent on the organism and antibiotic tested. Unfortunately, differences in national clinical and microbiological data can result in different breakpoint recommendations for the same species[92]. Microorganisms with a susceptibility between S and R levels are intermediately resistant, but not listed as a separate category.

The EUCAST introduced the epidemiological cut-off value (ECOFF) for situations in which clinical breakpoints have not been assessed[91,92]. A microorganism is defined as wild type for a species by the absence of phenotypically detectable acquired resistance mechanisms to the agent in question. The MIC or zone diameter distribution for a collection of organisms is described as a wild type MIC or zone diameter distribution. The ECOFF value is the highest reported wildtype MIC for an organism[93]. EUCAST also keeps track of these values and publishes guidelines for the determination of breakpoints for antibiotic resistance[94].

The most applied method to determine antibiotic resistance is by phenotypical testing. Various automated systems exist and have been approved by the Food and Drug Administration (FDA)[95]. These systems measure the growth of bacteria in the presence of various concentrations of different antibiotics in broth. The automated systems include the MicroScan WalkAway (Beckman Coulter, Brea, U.S.A.), Vitek 2 (bioMérieux, Marcy-l'Étoile, France), BD Phoenix Automated Microbiology System (Becton Dickinson, Franklin Lakes, U.S.A.) and Sensititre (TREK Trek Diagnostic Systems, Sun Prairie, U.S.A.). The benefit of such systems is that they reduce the time and labour needed to screen antibiotic susceptibility, thereby increasing throughput and reducing costs. However, the systems do not determine the actual MIC and an external confirmation is necessary in case of suspected antibiotic

resistance[96]. Confirmation of antibiotic resistance can then be achieved with a variety of phenotypic test, such as disk diffusion and dilution methods. Disk diffusion determines the MIC by applying filter paper disks with a fixed antibiotic concentration to an agar plate[1]. The antibiotic will then diffuse through the plate, resulting in a concentration gradient across the plate. An inoculated bacterium will grow towards the area where an inhibitory concentration of antibiotic is present. This inhibition zone can then be reported as the MIC. In dilution methods, bacteria are grown in the presence of either standardized broth or agar with different concentrations of antibiotic. The lowest concentration which does not result in a visible growth is then reported as the MIC[1]. The disk diffusion and dilution methods are still the gold standard for phenotypical testing.

Rapid detection of beta-lactamases

As mentioned above, the most prevalent form of antibiotic resistance towards beta-lactams is by the activity of beta-lactamases. Genotypic testing is performed to detect the presence of beta-lactamase genes. Polymerase chain reaction (PCR) assays are frequently employed for beta-lactamase detection and are available for many types, such as AmpC beta-lactamases[97], ESBLs[98] and carbapenemases. There are also multiplex variants where multiple beta-lactamase types are evaluated simultaneously[99-102]. Variations in the genes such a single-nucleotide polymorphisms (SNPs) may interfere with PCR analysis if they are in the primer region. Moreover, certain SNPs may be missed when PCR is not followed by sequencing. This can be especially problematic in the case of beta-lactamases such as the SHV class, where a single point mutation can change the spectrum of activity to include an entire class of antibiotics[103]. Additionally, the presence of a resistance gene does not necessarily result in production of the encoded protein and the resistant phenotype.

New functional assays for the detection of beta-lactamases have been described in recent years[104]. These tests evaluate the conversion of substrates and are therefore specific for the mode of beta-lactam resistance. For extended-spectrum beta-lactamases (ESBLs), this includes the Rapid ESBL NDP test, which detects the pH shift resulting from antibiotic hydrolysis using a colour indicator[105]. Hydrolysis of the carbapenem transforms the ring into an acidic group, which results in a decrease in pH which is shown using an indicator. The β-lacta for the detection of ESBLs works in a similar manner, however the target here is a chromogenic substrate that changes colour when hydrolysed[106]. These tests are optimized for the detection of ESBLs and are useful as a supplement to traditional susceptibility testing, but provide no insight beyond the phenotype.

Phenotypical detection of carbapenemases

In case that reduced susceptibility to carbapenems is suspected, this should be confirmed using a phenotypical test. The classic methodology for this is the disk diffusion method. In this test bacteria are plated on a disk with areas of defined antibiotic concentration. The

microorganism will then grow until the zone where an inhibitory concentration of antibiotic is present. EUCAST has developed disk diffusions test that comply with their own defined MIC breakpoints (http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/). The combination disk tests consists of discs with meropenem +/- various inhibitors (EUCAST) and have been well-validated in studies like the disk diffusion method. Boronic acid inhibits class A carbapenemases, dipicolinic acid inhibits class B carbapenemases. Cloxacillin inhibits AmpC β-lactamases and helps in excluding AmpC hyperproduction plus porin loss. However, these methods have a disadvantage in requiring incubation times of up to 18 hours, which has resulted in novel rapid methods being explored. The Modified Hodge Test (MHT) is also a culturing approach in which a carbapenem is inactivated by the resistant organism, which allows a susceptible indicator strain to grow. It has been widely used, but has significant drawbacks such as low specificity, varying performance with different types of carbapenemases and high numbers of false positives[107,108]. Media incorporating chromogenic substrates have also been developed for a variety of carbapenemases. CHROMagar KPC (CHROMagar Microbiology, Paris, France) has been reported as an efficient medium for detecting VIM and KPC carbapenemases[109]. Other examples include Brilliance CRE (Thermo Scientific, Hampshire, United Kingdom), ChromID CARBA (bioMérieux, Marcy-l'Étoile, France). These media typically perform well for class A and B carbapenemase, but struggle with OXA-48 types[110]. Another medium, the non-chromogenic Supercarba medium was developed by Poirel et al. specifically to detect low-level resistance carbapenemase producers such as OXA-48[109]. It appears to detect OXA-48 producers with a higher sensitivity than the commercially available chromogenic media[111]. However, the performance of all these media is highly variable and often struggle with lower level resistance organisms[112].

Alternative phenotypical testing systems have been developed and are based on detection of carbapenem hydrolysis. CarbaNP (I and II) and CarbAcineto have been developed and evaluated[113,114]. These developments have resulted in a commercial kit, RAPIDEC CARBA NP (bioMérieux, Marcy-l'Étoile, France)[115]. Alternative kits such as Rapid CARB Screen Kit and Blue- Carba are also available[116]. The principle of these tests is that carbapenem hydrolysis will give rise to a pH-change which will result in a colour change from red to yellow with phenol red solution. These kits are excellent tools for rapid screening due to their ease, low cost and sensitivity but when comparing the commercially available kits, it becomes clear that their performance is not optimal, especially with OXA-48 like carbapenemases[117]. While the performance of the kits is similar, in various comparisons the Carba NP test generally performs slightly better and would be the preferred choice[117,118]. Their limitations with regards to the detection of low level carbapenemase producers and inability to distinguish between carbapenemase types means that additional testing is still a requisite[112].

Mass spectrometry–based identification of antibiotic resistance detection

Considerable efforts have been undertaken to use MALDI-TOF platforms for detection of beta-lactamases. The platform has been applied to monitor substrate hydrolysis directly, by identifying signals corresponding to the antibiotic and its breakdown product. For extended-spectrum beta-lactamases this has been performed with cefotaxime and ceftazidime conversion[119]. While the technique was successfully tested in a proof-of-principle experiment, a routine implementation in microbiological laboratories has proven difficult. This is due to the pitfalls of interpreting "intermediate" test results, where the difference between susceptible and resistant isolates are difficult to assess against a background of background hydrolysis. Significantly more effort has been spent on applying this technique for the detection of carbapenemases. All the major carbapenems have been tested, with publications on meropenem[120,121], imipenem[122-124] and ertapenem[125,126], in bacteria like *E coli*, *K. pneumoniae, P. aeruginosa* and *A. baumannii*. The different levels of carbapenemase activities, combined with natural instability for some of these antibiotics complicates accurate resistance characterisation[127]. This has resulted in a multitude of protocols and approaches with no single established or reference method. Further standardisation will be key to transform this kind of hydrolysis assay into a reliable tool[128].

Direct identification of proteins offers a complete new avenue for antibiotic resistance assessment. The most common approach for protein identification using mass spectrometry is bottom-up proteomics, where proteins are enzymatically digested using proteases to create peptide mixtures. Trypsin is the most commonly employed protease. Trypsin cuts after every lysine (K) or arginine (R) that is not adjacent to a proline (P). Due to this specificity, it yields predictable peptides, with a size that is preferable to most conventional mass spectrometers. Alternative proteases such as chymotrypsin, LysC or ArgC or a combination of those may be used if more extensive digestion is required[129]. Of note is the fact that while an increase in number of unique, detectable, peptides may extend the total coverage of the proteome, it also enhances method complexity both from an analytical and data interpretation point of view and does not necessarily yield improved results. The field of proteomics has made such progress in the past two decades, resulting in comprehensive proteomics (i.e. towards full proteome coverage) becoming a reality. Early reports showed that such studies can yield valuable insight into antibiotic resistance related proteins, by identifying a variety of resistance associated proteins[130,131]. Now, with even further technological advancements being made, quantitative comparisons between proteomes belonging to different strains (i.e. resistant and susceptible strains) can be made directly. Such analyses are being used to establish known and new resistance mechanisms across the proteome[132,133].

Clearly, proteomics is a very powerful tool to obtain more insights in antibiotic resistance mechanisms. Beta-lactamases in particular are an attractive target, as their presence directly affects antibiotic susceptibilities.

Scope of the thesis

The overall objective of this study is to develop and test mass spectrometry based methodologies for the analyses of bacterial proteins which are associated with antibiotic resistance and/or can be used for typing of bacteria.

Chapter 2 describes a novel bottom-up proteomics workflow, utilising CE-ESI-MS/MS to identify carbapenemases in Gram-negative bacteria. The method was developed and tested on recombinant beta-lactamase as well susceptible and resistant *Escherichia coli* lab strains. The optimized method was evaluated using a collection of carbapenemases producing clinical isolates.

Chapter 3 presents a liquid chromatography tandem mass spectrometry (LC-MS/MS) platform for the identification of extended-spectrum beta-lactamases directly from positive blood culture bottles. For this study positive blood cultures were collected prospectively in two academic hospitals. Proteomic analysis of these isolates was performed and compared to phenotypic testing and PCR analysis.

Chapter 4 evaluates the use of matrix-assisted laser desorption/ionization Fourier-transform ion cylcotron resonance mass spectrometry (MALDI-FTICR MS) for ultrahigh resolution profiling of a collection of multidrug resistant *Pseudomonas aeruginosa* strains. The performance of the method is evaluated by comparing the results with a genotypic standard (AFLP).

Chapter 5 details the discovery of a novel carbapenemase in *Achromobacter xylosoxidans*. A susceptible and a resistant strain that developed under carbapenem treatment were investigated using comparative proteomic analysis. A new carbapenemase was found and further studied by heterologous expression in a susceptible *E. coli* strain.

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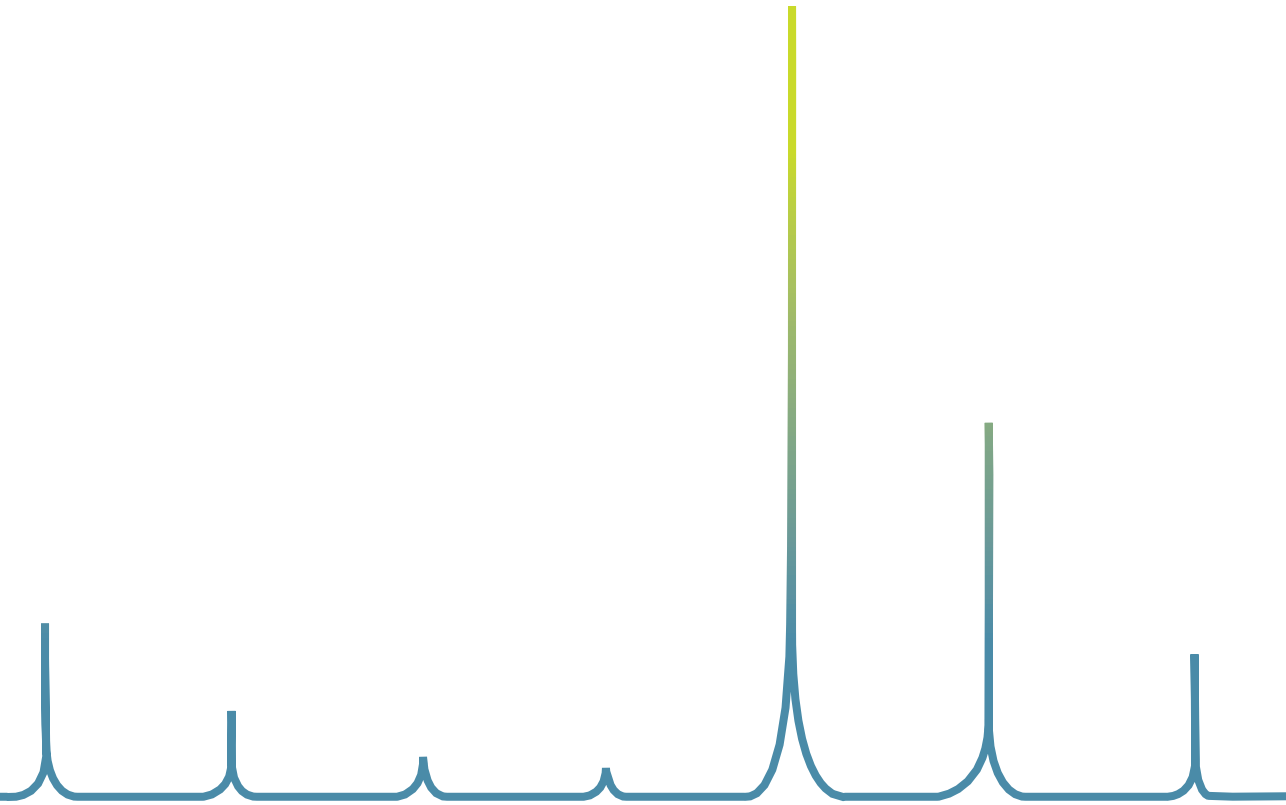

CHAPTER 2

Capillary–electrophoresis mass spectrometry for the detection of carbapenemases in (multi–) drug–resistant Gram–negative bacteria

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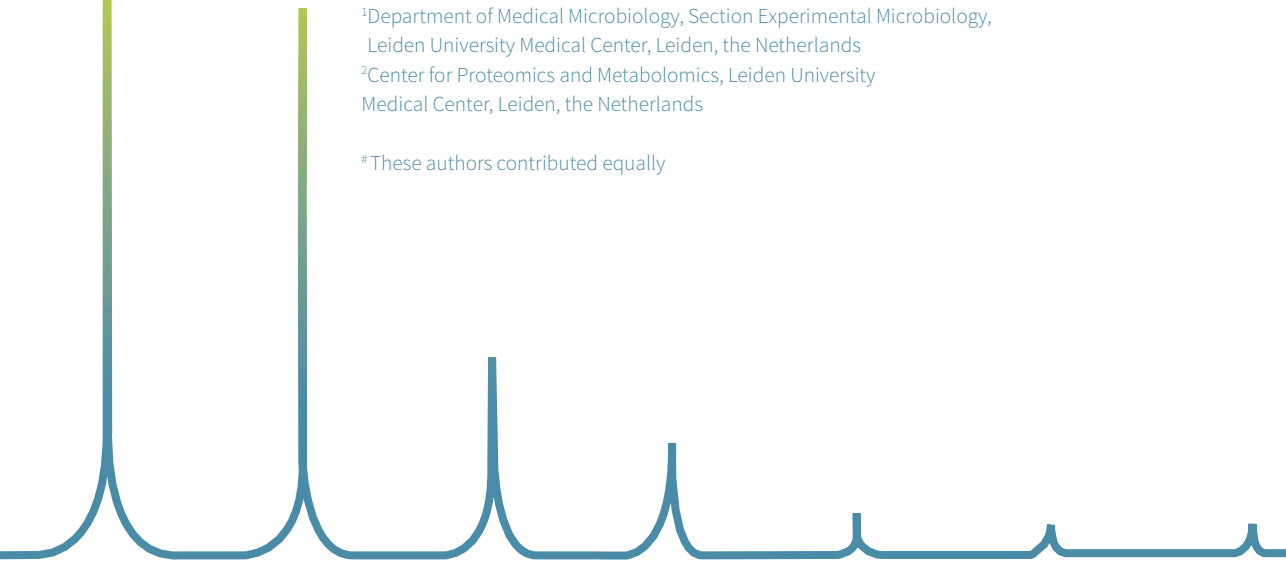

Abstract

In a time in which the spread of multi-drug resistant microorganisms is ever increasing, there is a need for fast and unequivocal identification of suspect organisms to supplement existing techniques in the clinical laboratory, especially in single bacterial colonies. Mass-spectrometry coupled with efficient peptide separation techniques offer great potential for identification of resistant-related proteins in complex microbiological samples in an unbiased manner.

Here we developed a capillary electrophoresis-electrospray ionization-tandem mass spectrometry CE-ESI-MS/MS bottom-up proteomics workflow for sensitive and specific peptide analysis with the emphasis on the identification of β-lactamases (carbapenemases OXA-48 and KPC in particular) in bacterial species. For this purpose, tryptic peptides from whole cell lysates were analysed by sheathless CE-ESI-MS/MS and proteins were identified after searching of the spectral data against bacterial protein databases.

The CE-ESI-MS/MS workflow was first evaluated using a recombinant TEM-1 β-lactamase, resulting in 68% of the amino acid sequence being covered by 20 different unique peptides. Subsequently, a resistant and susceptible *Escherichia coli* lab strain were analysed and based on the observed β-lactamase peptides, the two strains could easily be discriminated. Finally, the method was tested in an unbiased setup using a collection of in-house characterised OXA-48 (n=17) and KPC (n=10) clinical isolates. The developed CE-ESI-MS/MS method was able to identify the presence of OXA-48 and KPC in all of the carbapenemase positive samples, independent of species and degree of susceptibility. Four negative controls were tested and classified as negative by this method. Furthermore, a number of extended-spectrum beta-lactamases (ESBL) were identified in the same analyses, confirming the multi-resistant character in 19 out of 27 clinical isolates. Importantly, the method performed equally well on protein lysates from single colonies. As such, it demonstrates CE-ESI-MS/MS as a potential next generation mass spectrometry platform within the clinical microbiology laboratory.

Introduction

Antibiotic resistance is a growing problem of modern medicine. Micro-organisms that are resistant to a range of antibiotics are becoming widespread and at the same time the development of novel antibiotics is slowing down[1]. One of the ways to respond to the increase of multidrug resistant microorganisms is to ensure a fast identification of suspect organisms to allow for appropriate infection prevention strategies. As an example, for hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) appropriate screening methods have proven to be a key factor in the decline in prevalence in recent years[2].

One of the most important antibiotic classes with a broad range of antimicrobial activity is the β-lactam group, all sharing a structural similarity in possessing the β-lactam ring. They act by inhibiting enzymes involved in cell wall synthesis which eventually results in cell death[3]. They are widely applied and as a result there has been an increase in resistance to this class of antibiotics. Resistance to β-lactam antibiotics is predominantly caused by the expression of β-lactamases, which hydrolyse the β-lactam ring rendering the antibiotic inactive[4]**.** The carbapenem class of antibiotics is a keystone in the treatment of bacteria that have developed a resistance against other type of β-lactam antibiotics through extended-spectrum β-lactamases (ESBLs)[5]. Carbapenem resistance in turn is now developing and becoming more widespread. The most prevalent carbapenemases are KPC, VIM, IMP, NDM-1 and OXA-48[6]. OXA-48 and *Klebsiella pneumoniae* carbapenemases (KPC) are two important carbapenemases that are found in various bacterial species[7]. OXA-48 has first been identified in Turkey in 2001 and has proliferated ever since. Recent years have seen outbreaks of OXA-48 positive bacteria in a number of countries[8].

The proteomic fingerprinting using MALDI-ToF MS has been implemented in many medical microbiological laboratories as a tool for the identification of bacteria[9]**.** However, this approach is usually insufficient for direct identification of resistant phenotypes and is not suited for confident identification of the specific proteins accountable for this resistance. For this purpose, true identification of proteins involved in resistance, such as beta-lactamases, is necessary. Bottom-up proteomics analysis using liquid chromatography coupled to mass spectrometry is a very well accepted approach for this kind of analyses and has recently also been used to study β-lactam resistance in *Acinetobacter baumannii*[10]. An alternative technical solution could be capillary electrophoresis (CE). CE is a highly efficient method for peptide analysis since it is able to separate and analyse peptides with a wide range of physico-chemical properties, and compared to liquid chromatography-mass spectrometry (LC-MS), is particularly suited for the analysis of smaller peptides[11]. The recently developed innovative interfaces (both employing sheath-liquid and sheathless variants) have considerably advanced the coupling of CE with MS with the key benefit of the possibility to work at ultra-low flow rates, resulting in reduced ion suppression and improved sensitivity[12,13].

The analytical parameters and advantages of such methods for peptide analysis have recently been evaluated[14,15].

All these aspects indicate that CE-MS is a potentially valuable technique for confident identification of, and differentiation between, specific beta-lactamases in bacteria. However, the real application and performance of such a method in the analyses of clinically relevant samples is still lacking. The aim of this study was to develop a CE-ESI-MS/MS based method as well as compatible sample preparation for the identification of beta-lactamases in complex samples. Because of the difficulty with current methodologies to identify OXA-48 and KPC producing *Enterobacteriaceae*[16], we decided to test the method on a well-defined set of such clinical isolates.

Materials and methods

Characterization of the reference set of Gram–negative clinical isolates

The collection of Gram-negative clinical isolates (*Klebsiella pneumoniae*, *Escherichia coli* and *Enterococcus cloacae*) consisted of isolates obtained in-house from patients admitted to the hospital as well as a number of isolates from a reference set of the Dutch National Institute for Public Health and Environment (RIVM, Bilthoven, the Netherlands). The meropenem minimum inhibitory concentration (MIC) was determined using an E-test according to manufacturer's instructions (Biomérieux Benelux, Zaltbommel, the Netherlands). Screening for carbapenemase production was performed by a modified Hodge-test, as previously described by Lee[17]. All isolates were characterized at the molecular level; most OXA-48 isolates were characterized by a PCR assay at the RIVM. One was characterized by PCR at the carbapenemase reference laboratory of P. Nordmann (South-Paris Medical School, Paris, France) and one was characterized by MicroArray (Checkpoints, Wageningen 4, the Netherlands). All KPC isolates were characterized by a real-time in-house PCR assay at the LUMC.

MALDI-ToF MS analysis of carbapenem breakdown was performed according to the protocol as described by Sparbier[18] with slight modifications. In short: bacteria were cultured for 18-24 hours on Columbia blood agar plates (Biomerieux Benelux, Zaltbommel, the Netherlands). The amount of bacteria filling an 1 µl inoculation loop were suspended in 30 µl ertapenem (Invanz®, Merck, Haarlem, the Netherlands), 0.5 mg/ml in 10 mM ammonium hydrogen citrate (AHC), pH 7) and incubated for 3 hours. After centrifugation for 2 min at 13.000 x *g,* 1 µL of the cell-free supernatant was spotted onto a steel polished MALDI plate in triplicate and dried at room temperature. Subsequently the spot was covered with 1 µl α-HCCA in 50% acetonitrile-2.5% trifluoroacetic acid. Each run included a negative control (*E. coli* ATCC 25922 incubated in the presence of ertapenem) and an ertapenem control (ertapenem in

AHC), both pre-treated as above. Fifteen carbapenemase negative strains from the American Type Culture Collection (ATCC) were also tested.

Spectra were obtained with a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany). The spectrum per spot was the product of 240 shots. Spectra were measured in positive linear mode in a mass range van 100 to 1100 Da. Spectra were analysed using FlexAnalysis 3.3 (Bruker Daltonics) in the range from *m/z* 440 to 580. Successful identification of carbapenemase activity was defined as complete disappearance of peaks at *m/z* 475.5, *m/z* 498.5 and *m/z* 520.519.

Cell culturing, lysis and in–solution protein digestion using trypsin

Cells were grown in Brain Heart Infusion broth (Biomérieux Benelux, Zaltbommel, the Netherlands) and pellets from 1 ml cultures after 1 min 10.000g were re-suspended in a fixed volume of deionized water. Total protein content in these samples was analysed with the Pierce Micro BCA Protein Assay kit (Thermo Scientific), following the manufacturer's instructions. The samples were then stored at -80 °C until further use.

For the lysis and digestion of the samples an adapted protocol based on the protocol by Wang et al.[20] was used. In short, a cell suspension corresponding to 20 µg of protein content was diluted 1:1 (v/v) in trifluoroethanol (TFE). The sample was vortexed and incubated at 60 °C for 1 hour. The sample was then sonicated in a water bath for 2 min. Ammonium bicarbonate and dithiothreitol (DTT) were added with final concentrations of 25 mM and 2.5 mM respectively and the sample was reduced at 55 °C for 15 min. For the alkylation, iodoacetamide (in 25 mM ammoniumbicarbonate) was added to final concentrations of 5.5 mM. Alkylation took place in the dark at room temperature for 15 min. Trypsin was added to the sample at a 1:20 enzyme to protein ratio and the sample was digested overnight at 37 °C. The digestion was quenched with 10% acetic acid and the samples were lyophilized and subsequently reconstituted in 1% acetic acid and 50 mM ammonium acetate pH 4.

For the analyses of single bacterial colonies, cells were grown on Columbia blood agar plates as described above. Bacterial colonies were picked individually by re-suspending them in 20 µl of deionized water. Then, 20 µl of TFE was added and lysis and tryspin digestion was subsequently performed as described above.

CE–ESI–MS(/MS) analysis

Capillary-electrophoresis mass spectrometry analysis was performed on a PA800 plus (Beckman Coulter, Brea, CA) coupled to a maXis Impact UHR-TOF-MS (Bruker Daltonics) utilizing an interface based on the initial design by Moini[21]. Prior to analysis, the capillary was sequentially rinsed with 0.1 M NaOH, 0.1 M HCl, deionized water and finally the background electrolyte, which consisted of 10% acetic acid. Samples were hydrodynamically injected at

1 psi for 60 s, which results in a sample plug equivalent to 1% of the total capillary volume. The separation was performed at 20 kV for 60 min using a bare fused-silica capillary. Eluting peptides were analyzed using the data dependent MS/MS mode over an *m/z* 300-2000 range. The 10 most abundant ions in an MS spectrum were selected for MS/MS analysis by collision-induced dissociation using helium as the collision gas. A 1 minute dynamic exclusion window was used for precursor selection and the window for fragment analysis ranged from *m/z* 300-2200.

Data analysis

A database was prepared using a collection of gram-negative bacteria. The databases were obtained from http://org.uib.no/prokaryotedb/[22]. This final database was supplemented in-house with various β-lactamase sequences. Peak lists were generated using Data analysis 4.0 (Bruker Daltonics, Bremen, Germany) with default settings and exported as Mascot Generic Files. Peptides were identified using the Mascot algorithm (Mascot 2.4.1, Matrix Science, London, UK) using Mascot Deamon 2.2.2. A MS tolerance of 0.05 Da and a MS/MS tolerance of 0.8 Da were used. Trypsin was designated as the enzyme and up to one missed cleavage site was allowed. Carbamidomethylcysteine was selected as a fixed modification and oxidation of methionine as a variable modification. The false discovery rate (peptide matches above the identity threshold) was set at 1% using a decoy database. For the scoring of beta-lactamases, only beta-lactamase hits with at least two unique peptides with a score above 25 were selected.

Results

CE–ESI–MS(/MS) analysis of recombinant TEM beta–lactamase and ampicillin–resistant and susceptible E. coli laboratory strains

The performance of the method was tested using a tryptic digest of recombinant TEM-1 beta-lactamase. Using 1 ng of protein, TEM-1 was identified with 20 unique peptides and a resulting total sequence coverage of 68% (data not shown). To test the applicability of detecting this beta-lactamase in a complex sample, an ampicillin resistant *E. coli* lab strain (BL21(DE3)) containing a plasmid encoding a TEM β-lactamase was tested along with the same ampicillin sensitive strain lacking this plasmid. Hence, the only difference between the two strains is the expression of β-lactamase in the resistant strain. Tryptic digests of bacterial lysates were analysed in triplicate by CE-ESI-MS (Figure 1A). Based on the *in-silico* tryptic digest of TEM ß-lactamase, the CE-ESI-MS runs were screened for the presence or absence of the predicted ß-lactamase derived tryptic peptides as exemplified by several extracted ion chromatograms shown in Fig. 2B and C.

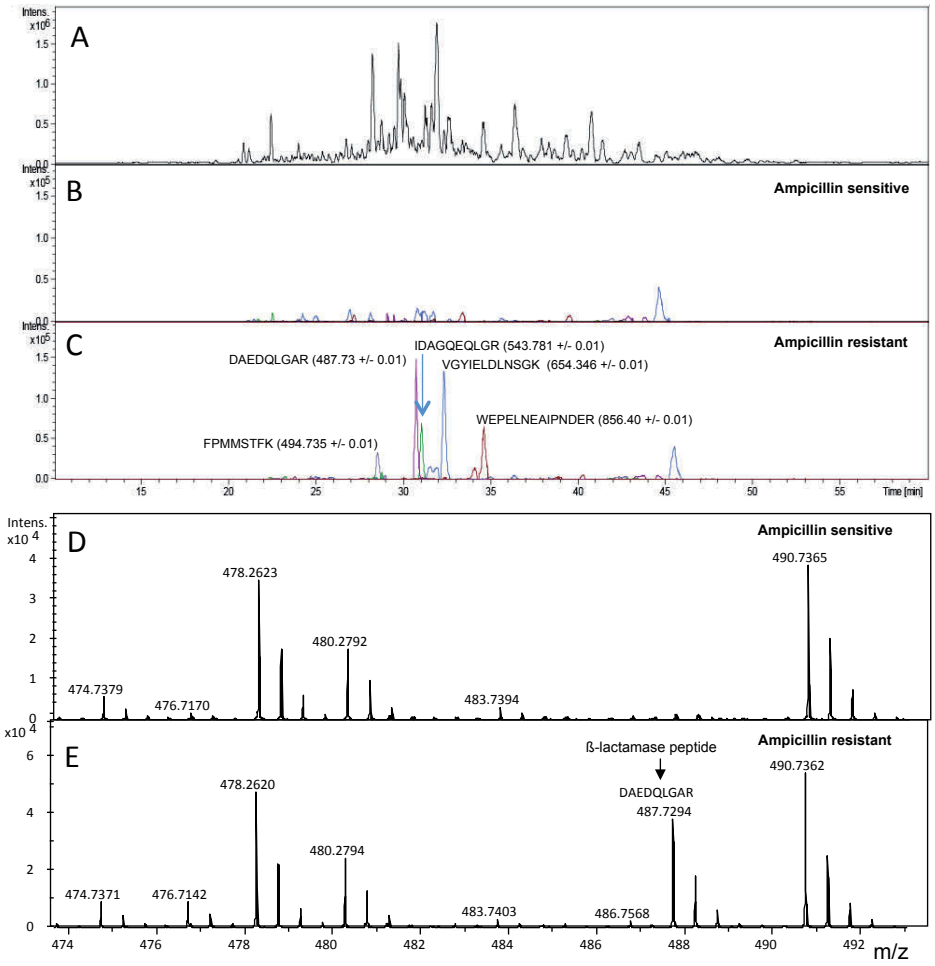

Figure 1: CESI–MS/MS analysis of a tryptic digest of an ampicillin sensitive and resistant *E. coli* strain.

A: Representative base peak electropherogram as observed for an E. coli tryptic digest. B and C: Extracted ion chromatograms (EIC) of several *m/z* values corresponding to tryptic peptides of beta-lactamase that are absent in the ampicillin sensitive strain (B) but present in the resistant strain (C). D and E: Mass spectrum at a fixed time point (30.3 min) showing the β-lactamase peptide DAEDQLGAR (*m/z* 487.7294) in the resistant strain (E) which does not appear in the sensitive strain (D). The other peaks within the spectrum correspond to non-β-lactamase peptides, shared by the resistant and sensitive strain.

Table 1 Overview of the clinical isolates used in this study. Listed are the carbapenemases (KPC and OXA–48) as identified by PCR, the results of the modified Hodge test, an E–test (Minimal Inhibitory Concentration (MIC)) and the MALDI–ToF MS ertapenem breakdown analysis. In the last column the results of the bottom–up proteomics analysis by CESI–MS/MS are listed showing that all target β–lactamases are identified as well as a number of extended–spectrum β–lactamases. See material and methods for further details.

Carbepenemase (PCR)	Species:	Hodge test:	Meropenem (MIC in mg/L)	MALDI-ToF MS (Ertapenem degradation)	CESI-MS/MS (bottom-up proteomics)
KPC	*K. pneumoniae*	pos	>32	ID +	KPC
KPC	*K. pneumoniae*	pos	> 32	ID +	KPC
KPC	*K. pneumoniae*	pos	2	ID +	KPC/SHV
KPC	*K. pneumoniae*	pos	2	ID +	KPC
KPC	*K. pneumoniae*	pos	32	ID +	KPC/SHV
KPC	*K. pneumoniae*	pos	32	ID +	KPC/SHV
KPC	*K. pneumoniae*	pos	12	ID +	KPC/TEM/SHV
KPC	*K. pneumoniae*	pos	> 32	ID +	KPC/SHV
KPC	*K. pneumoniae*	pos	3	ID +	KPC
KPC	*K. pneumoniae*	pos	6	ID +	KPC
OXA-48	*K. pneumoniae*	pos	24	ID -	OXA-48/CTX
OXA-48	*K. pneumoniae*	pos	8	ID -	OXA-48/CTX/SHV
OXA-48	*K. pneumoniae*	pos	1,5	ID +	OXA-48/CTX
OXA-48	*K. pneumoniae*	pos	> 32	ID -	OXA-48
OXA-48	*K. pneumoniae*	pos	2	ID -	OXA-48/TEM
OXA-48	*E. coli*	pos	0,5	ID -	OXA-48/CTX
OXA-48	*K. pneumoniae*	pos	1	ID -	OXA-48/CTX
OXA-48	*K. pneumoniae*	pos	3	ID -	OXA-48/CTX
OXA-48	*K. pneumoniae*	neg	0,4	ID +	OXA-48
OXA-48	*K. pneumoniae*	pos	2	ID +	OXA-48
OXA-48	*K. pneumoniae*	pos	24	ID -	OXA-48/CTX
OXA-48	*E. cloacae*	pos	1,5	ID -	OXA-48/CTX
OXA-48	*E. cloacae*	pos	0,5	ID -	OXA-48/ampC
OXA-48	*K. pneumoniae*	pos	>32	ID -	OXA-48/TEM
OXA-48	*K. pneumoniae*	pos	32	ID -	OXA-48/CTX/SHV
OXA-48	*K. pneumoniae*	pos	0,5	ID -	OXA-48/CTX
OXA-48	*K. pneumoniae*	pos	0,5	ID -	OXA-48/CTX

For example, the tryptic ß-lactamase peptide DAEDQLGAR (*m/z* 487.729 $[M+2H]^{2+}$) was examined. In the extracted ion chromatogram of ions with *m/z* 487.73 ±0.01 a clear peak is visible in the run of the ampicillin resistant strain which is absent in the sensitive strain (Compare figures 1B and C). Close inspection of the underlying MS spectra (Figure 1D and 1E) indeed shows that amongst other peptides that are co-migrating with this peptide, which are identical in both runs, a clear signal at *m/z* 487.7294 is present in the spectrum derived from the resistant strain, corresponding to the above mentioned peptide DAEDQLGAR (theoretical *m/z* 487.7305). In total, 14 of such differential peptides with masses corresponding to ß-lactamase tryptic peptides were identified in all CE-ESI-MS runs from the ampicillin resistant strain, while none of these were identified in the non-resistant strain. The nature of the TEM beta-lactamase peptides was confirmed by MS^2 analysis (data not shown).

Selection and characterization of clinical isolates

To test the performance of our method for the identification of OXA-48 and KPC carbapenemases, a collection of clinical isolates containing such carbapenemases was needed. For this purpose, a set of samples were selected that were PCR positive for either of these carbapenemases (Table 1). For phenotypic characterization, a modified Hodge test for the detection of carbapenemases was used which showed that all isolates, but one, were positive (Table 1). In addition, the meropenem minimal inhibitory concentration (MIC) was determined using an E-test as a measure of the degree of carbapenem resistance. This showed that the set of isolates varied considerably in their degree of resistance to meropenem (Table 1.) Finally, all KPC isolates were positively identified (10/10) using the MALDI-ToF MS ertapenem hydrolysis assay. However, with this assay only 3/17 of OXA-48 isolates were positively identified (Table 1). A tenfold dilution of ertapenem (0.05 mg/ml) using a subset of samples increased detection to 9 out of 13 isolates without loss in specificity (i.e. ertapenem peaks were still present in all 15 carbapenemase negative strains). Overnight incubation affected specificity as only 5/15 negative strains were still correctly identified.

Analysis of clinical isolates using CE–ESI–MS/MS

The collection of clinical isolates was analysed in triplicate using the CE-ESI-MS/MS platform. Fragmentation spectra were acquired in a data dependent manner and a MASCOT database search was performed to identify peptides based on the recorded spectra. Importantly, for confident identification of specific beta-lactamases subspecies, unique discriminating peptides need to be detected. A phylogenetic tree based on a sequence alignment using a selection of enzymes covering the various classes of beta-lactamases shows that the differences in the primary amino acid sequences within a certain class may be very small (e.g. the KPC group) while for other classes (e.g. the OXA group), differences in the primary structures are more pronounced (Figure 2A). As such, the discrimination between different classes can be made based on multiple different tryptic peptides, while identification of a single type may rely on an individual peptide. In practice, the lactamases were identified

with a minimum of 4 different peptides using a merged database search of triplicate analysis although all individual analyses yielded lactamase identifications as well. In the case of KPC, the class assignment was confidently performed based on several unique peptides (an example shown in Figure 2B) while within the OXA–group, OXA-48 could be unambiguously identified (Figure 2C).

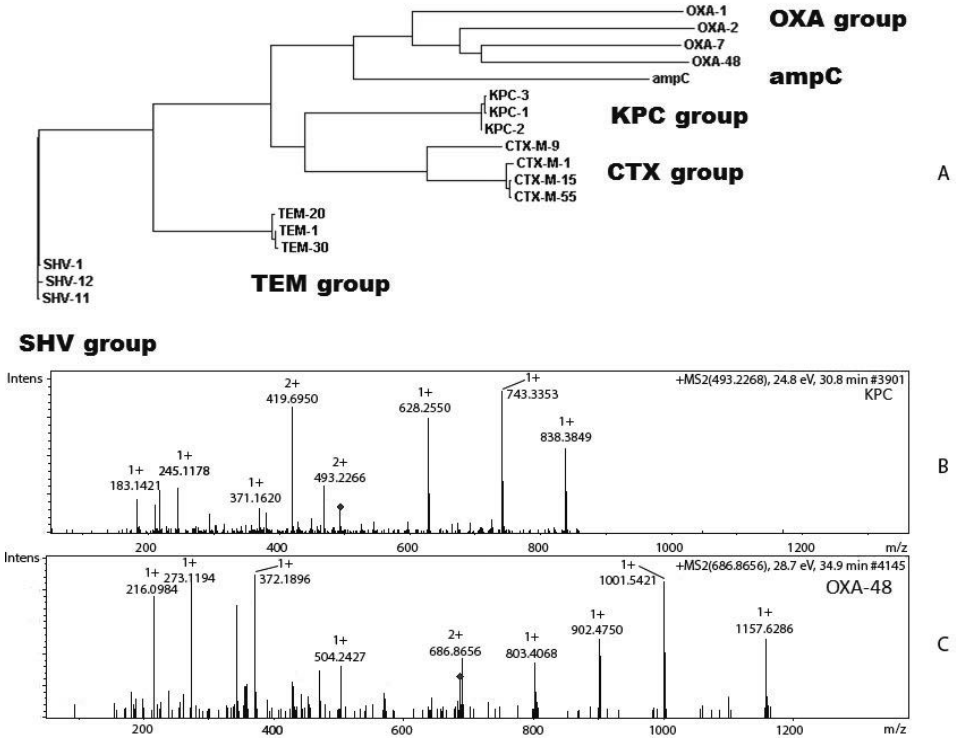

Figure 2: Differentiation between different β–lactamase subgroups and species.

A: Phylogenetic tree based on a sequence alignment of different β-lactamases from different functional subgroups. For some classes the primary structures can be highly similar (e.g. KPC and CTX) while for others, individual sequences can vary considerably (e.g. OXA). B: Tandem mass spectrum for a unique tryptic peptide from the KPC-group of carbapenemases (FPLCSSFK). C Tandem mass spectrum for a unique tryptic peptide from OXA-48 (SQGVVVLWNENK).

A

OXA-48

```
1       MRVLALSAVFLVASIIGMPAVAKEWQENKSWNAHFTEHKSQGVVVLWNEN
51      KQQGFTNNLKRANQAFLPASTFKIPNSLIALDLGVVKDEHQVFKWDGQTR
101     DIATWNRDHNLITAMKYSVVPVYQEFARQIGEARMSKMLHAFDYGNEDIS
151     GNVDSFWLDGGIRISATEQISFLRKLYHNKLHVSERSQRIVKQAMLTEAN
201     GDYIIRAKTGYSTRIEPKIGWWVGWVELDDNVWFFAMNMDMPTSDGLGLR
251     QAITKEVLKQEKIIP
```

AA	Observed *m/z*	Mr(calculated)	Delta(ppm)	Score	Peptide
30 – 39	628.792	1255.573	-3	45	SWNAHFTEHK
40 – 51	686.865	1371.715	0.4	61	SQGVVVLWNENK
52 – 60	525.272	1048.530	-1	50	QQGFTNNLK
95 – 100	381.680	761.346	-1.4	20	WDGQTR
101 – 107	438.222	874.430	-1.3	37	DIATWNR
108 – 116	521.770	1041.528	-3.1	21	DHNLITAMK
117 – 128	729.377	1456.735	3.1	15	YSVVPVYQEFAR
164 – 174	632.848	1263.682	-1.5	69	ISATEQISFLR
175 – 180	401.731	801.450	-3.4	33	KLYHNK
181 – 186	370.706	739.340	0.4	42	LHVSER

B

Sample ID	Species:	Meropenem MIC mg/L	OXA-48 sequence coverage %
223 C1	*E. coli*	0.5	27
C2			32
C3			21
225 C1	*K. pneumoniae*	1	25
C2			29
C3			27
235 C1	*E. cloacae*	1.5	33
C2			34
C3			29
237 C1	*K. pneumoniae*	>32	31
C2			38
C3			38

Figure 3 CE–ESI–MS/MS identification of OXA–48 in tryptic digests derived from a single colony. Single bacterial colonies were sampled and processed for tryptic digestion, CE–ESI–MS/MS analysis and database searching.

A: Primary sequence OXA-48 and example of results from one CE-ESI-MS/MS analysis, showing multiple unique OXA-48 peptides with an overall sequence coverage (in red) of 33%. AA, amino acid number within the primary sequence. B: CE-ESI-MS/MS identification of OXA-48 in three individual colonies (C1-C3) from different OXA-48 positive bacterial species varying in the degree of susceptibility to meropenem.

Overall, using our CE-ESI-MS/MS platform, the presence of OXA-48 and KPC β-lactamases was demonstrated for all samples measured in triplicate (Table 1), as well as each analysis individually. This demonstrates that this method is capable to phenotypically identify OXA-48 and KPC positive clinical isolates. It is well known that within such isolates multiple different β-lactamases can be present which are often related to the multi-drug resistant phenotype of

such strains. An advantage of our method is that it is unbiased in nature and therefore may also reveal the presence of such resistance related enzymes. Indeed, within our set of clinical isolates a number of extended-spectrum beta-lactamases (ESBLs) such as CTX and SHV were observed (Table 1). 19 out of 27 of the clinical isolates were shown to harbour additional ESBLs as well as the known carbapenemases. A number of negative controls (n=4) were also analysed, in triplicate. These were all negative for beta-lactamases.

Analysis of single bacterial colonies

The applicability of our workflow would benefit from detection on colony level. Therefore, we tested the performance of our method on single colonies after growing on solid medium. Four isolates from the collection of OXA-48 positive strains were selected to be grown on blood agar plates overnight. From each plate three colonies were sampled and processed independently. Each colony was analysed in triplicate (4 ng of protein injected). Figure 3A shows the results obtained from one CE-ESI-MS/MS analysis of a colony from an OXA-48 clinical isolate, clearly demonstrating multiple OXA-48 unique peptides. Importantly, in all individual CE-ESI-MS/MS analyses of the independently sampled colonies OXA-48 was identified (Figure 3B). In addition, in several samples ESBLs were identified (data not shown). Overall, the OXA-48 sequence coverage varied between 21-38% in the different colonies.

Discussion

From the very early days of capillary electrophoresis the potential of the method for highly efficient separation of peptides and proteins was commonly recognised[23-25]. Moreover, as a miniaturized technique operating at very low flow rates, CE represents a natural match for the ESI process providing advantages such as reduced ion suppression and improved sensitivity[12,26]. Yet, with the emergence of proteomics, liquid chromatography – mass spectrometry (LC-MS) became the mainstream method which pushed CE aside to a position of an interesting but exotic technique. A complex (in comparison to a RPLC-MS) interfacing between CE and MS partially explains the limited use of CE in proteomics. For instance, the most widely used interface, namely a sheath-liquid interface, is a technical compromise which provides a robust CE-MS hyphenation at the expense of sensitivity[27]. Recent years have seen a push into the development of novel CE-MS interfaces. As much as they are different in the technical details all those interfaces are being developed for a common purpose of maximising the natural advantages of the CE-MS as an ultra-low flow technique. Here, we present a CE-ESI-MS/MS based workflow for the identification of beta-lactamases in complex samples and demonstrate its applicability for the confident and sensitive analysis of carbapenemases (OXA-48 and KPC) in clinical isolates of Enterobacteriaceae. A sheathless porous sprayer interface was used to couple CE to the mass spectrometer, but we

are convinced that our approach can easily be translated to the set-ups using other CE-MS interfaces[28-30].

KPC is the most common carbapenemase found in the United States and has spread worldwide since its discovery in 1996, even becoming endemic in certain countries (e.g. Israel and Greece)[31]. KPC can be detected by the modified Hodge test and other phenotypic tests but these methods lack speed and can be difficult to interpret. OXA-48 also has become widespread worldwide but detection can be challenging. OXA-48-like producers exhibit a range of susceptibility patterns, and often show only decreased susceptibility to carbapenems, which renders standard susceptibility testing an insufficient screen. As opposed to other carbapenemase genes whose expression can be inhibited *in vitro* with certain compounds (e.g. EDTA, boronic acid), no phenotypic test can detect OXA-48-like producers in general. The modified Hodge test shows adequate performance[32] but requires an overnight incubation step and provides no information about the kind of carbapenemase that is present, which might prove essential in an outbreak-setting. Multiple PCR assays have been developed and have remained the gold standard for detection. However, clinical significance of the presence of OXA-48 like genes cannot be inferred from genotypic testing[33] and thus, the detection of the carbapenemase that is actually expressed would provide important information. Moreover, for the PCR-methods specific primers are needed, requiring *a priori* knowledge that may even become problematic in case specific mutations occur in the corresponding target sequences.

Our method (total analytical analysis time of 60 min, separation window of 30 min) enables the detection of carbapenemase in all tested colony samples with the sequence coverage between 21 and 38%. This is lower than the maximum sequence coverage that we have obtained with a purified beta-lactamase (68%), but sufficient for a confident identification and (depending on the beta-lactamase) assignment of the beta-lactamase class or even species discrimination. The diagnostic timeframe for our method in the current setting would be around 12 to 16 hours including tryptic digestion, which is longer than PCR. However, having established the analytical platform to identify carbapenemases now permits improvement of the digestion protocol, for example using microwave-assisted protein digestion methods which could potentially bring back the overall analysis time to 5-6 hrs[10].

As a result of the direct detection of the resistance linked enzymes, our CE-ESI-M/MS approach therefore offers an advantage over the methods described above. In every isolate analysed, the carbapenemase class as molecularly identified by PCR was also observed in CE-ESI-MS/MS analysis and was successful in different Gram-negative species. Since the method does not provide full sequence coverage, it will not be able to identify all individual β-lactamases, much as molecular techniques would do without additional sequencing steps. However, with the sequence coverage observed, the method still provides at least carbapenemase class identification. Moreover, the unbiased nature of the approach allows for the detection of a variety of β-lactamase enzymes without a requirement of pre-existing sample

knowledge. Thus, in addition to the carbapenemases confirmed through PCR, a number of extended spectrum β-lactamases were identified in the present study. The strains had not been previously tested for these other genes. The multi-resistant character of the isolates is not surprising; not only is multi-resistance widespread, it has also been documented that carbapenem resistance is commonly observed in species that already harbour resistance to other β-lactam based antibiotics through ESBLs[8].

Phenotypical tests such as E-tests can elucidate the degree of functional resistance per antibiotic[34]. The meropenem MIC concentration in the isolates selected for the current study ranged from 0.5 to over 32 mg/L, indicating a varying degree of susceptibility across the different isolates. The analysis by CE-ESI-MS/MS seemed to be independent of this however, performing similarly for highly resistant and still partially susceptible cells. The MALDI-TOF MS ertapenem breakdown assay, on the contrary, only correctly identified 3 out of 17 OXA-48 producers according to our definition, while KPC was easily detected with this method. Besides mechanistic explanations, such as kinetic differences between KPC and OXA-48 or availability of the ertapenem to the respective carbapenemase pool, the dependence of this method on the complete disappearance of product peaks in the mass spectrum also played a role. Very recently, a novel method to detect carbapenem breakdown with MALDI-ToF MS, using ertapenem impregnated disks, was described, showing a good performance, also with OXA-48 positive cells[35]. One potential problem with MALDI-TOF MS analysis of small molecules is related to the influence of the matrix effect. Therefore, we anticipate that CE-ESI-MS can potentially aid in this type of analysis, not only because of the elimination of the matrix effect, but also due to the separation of the substrate and products, resulting in more accurate detection and quantification capabilities. For similar reasons, several novel methods have recently been developed which allow a quantitative assessment of the carbapenem breakdown using single reaction monitoring on LC-ESI-MS/MS platforms[36,37].
Although not the major aim of the current experiments, our non-beta-lactamase proteomics data obviously also reveals the bacterial species (*K. pneumoniae*, *E. coli* or *E. cloacae*) harbouring the resistance phenotype. The total performance across an analysis generally results in 300-500 unique peptide identifications which lead to 100-200 protein identifications from as little as 10 ng of a tryptic digest. In other words, the analysis can combine species identification, similar as MALDI-ToF MS based approaches[38], but in addition reveals specific protein IDs and combines it with beta-lactamase resistance classification.

Obviously, more in-depth analysis, for example by using longer capillaries, capillaries with modified surface and/or multi-dimensional separations, may reveal other proteins involved in the resistance phenotype, such as proteins involved in drug uptake and excretion. However, such in-depth analysis is non-compatible with the throughput and speed of identification which the colony based protocol is demanding. Thus, as a next step, we are planning to

perform similar analyses in samples taken directly from blood cultures which would be the next step in speeding up the time till detection of the carbapenemase.

Conclusions

The growing incidence of β-lactam resistance asks for rapid, straightforward and clear identification of individual β-lactamases in clinical isolates to allow appropriate intervention strategies related to treatment and control of further spread. This study presents a novel CE-ESI-MS/MS workflow for the sensitive and unambiguous bottom-up proteomics identification of a OXA-48 and KPC in various Gram-negative species, even from single colonies. Additionally, due to the unbiased nature of the method, a number of non-targeted extended-spectrum β-lactamases were also identified, demonstrating the ability of the system to successfully assess multi-drug resistant bacteria. As such, it presents CE-ESI-MS as a potential next generation mass spectrometry platform for both species as well as resistance identification within the clinical microbiology laboratory.

Acknowledgements

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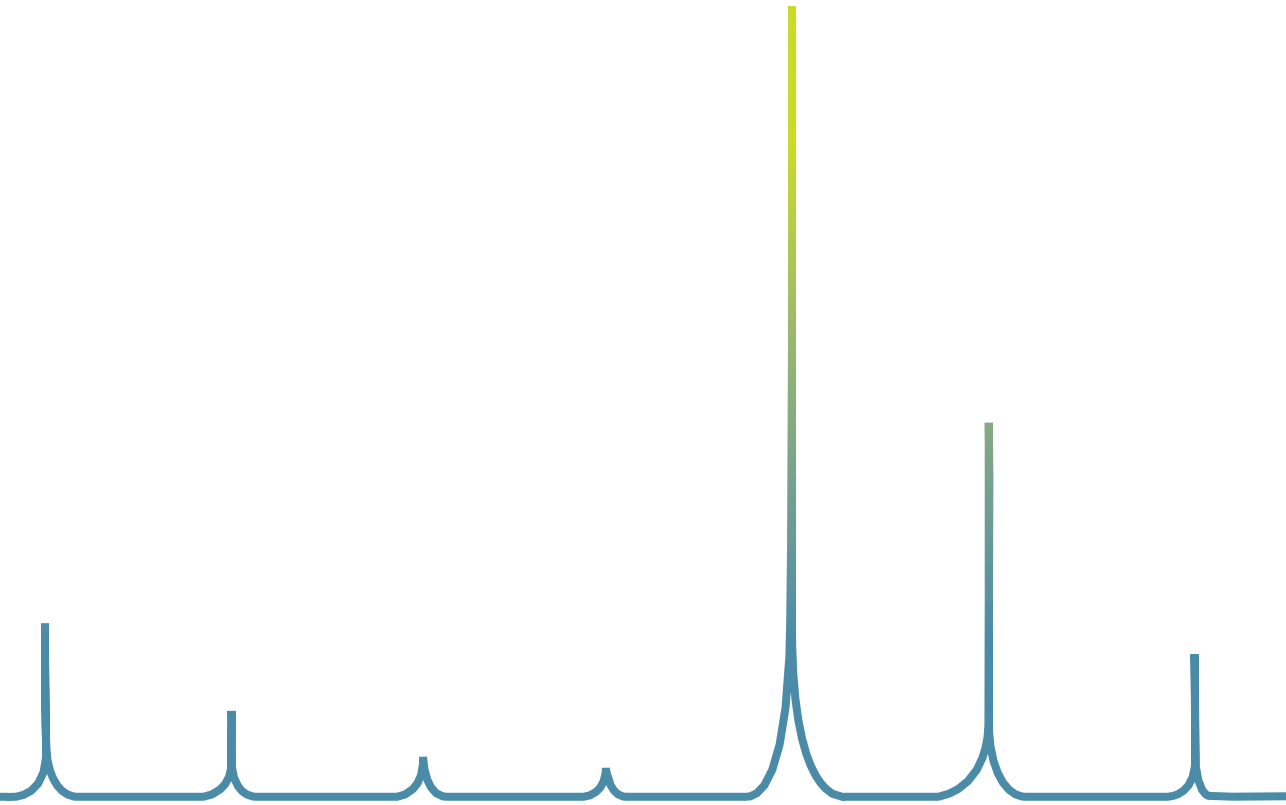

CHAPTER 3

Direct detection of extended-spectrum beta-lactamases (CTX-M) from blood cultures by LC-MS/MS bottom-up proteomics

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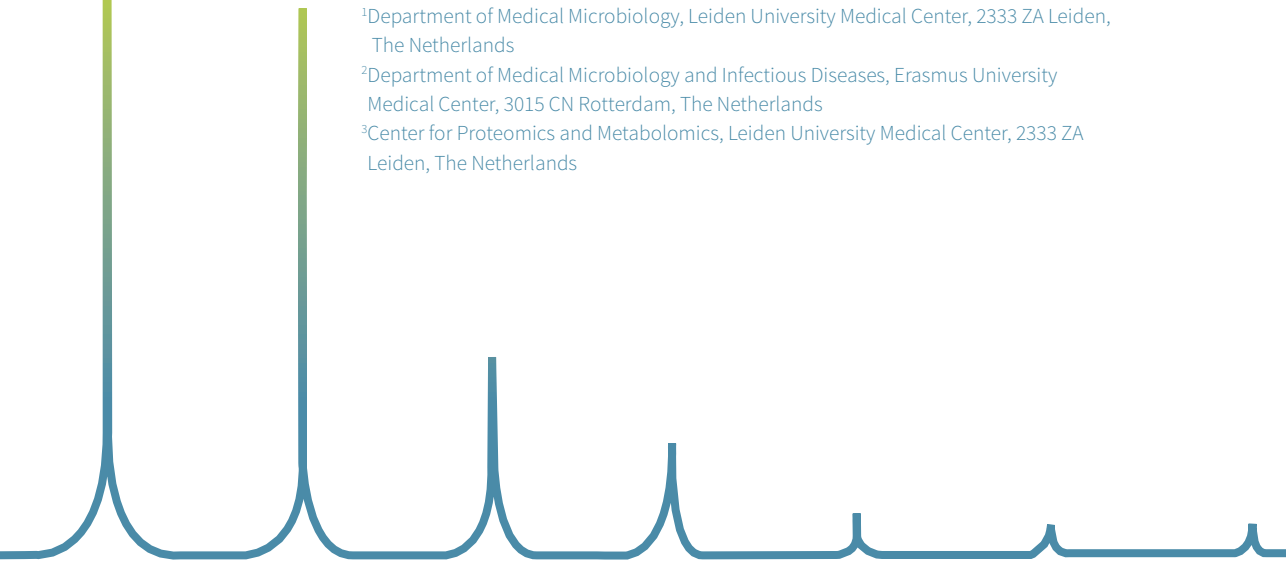

Abstract

Rapid bacterial species identification and antibiotic susceptibility testing in positive blood cultures have an important impact on the antibiotic treatment for patients. To identify extended-spectrum beta-lactamases (ESBL) directly in positive blood culture bottles, we developed a workflow of saponin extraction folllowed by a bottom-up proteomics approach using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The workflow was applied to positive blood cultures with *Escherichia coli* and *Klebsiella pneumoniae* collected prospectively in two academic hospitals over a four month period. Of 170 positive blood cultures, 22 (12.9 %) contained ESBL positive isolates based on standard susceptibility testing. Proteomic analysis identified CTX-M ESBLs in 95% of these isolates directly in positive blood cultures, whereas no false-positives were found in the non-ESBL producing positive blood cultures. The results were confirmed by molecular characterisation of beta-lactamase genes.

Based on this proof-of-concept study, we conclude that LC-MS/MS-based protein analysis can directly identify extended-spectrum beta lactamases in *E. coli* and *K. pneumoniae* positive blood cultures, and could be further developed for application in routine diagnostics.

Introduction

Infections caused by antibiotic resistant Gram-negative bacteria are an increasing problem worldwide. In the Netherlands, resistance towards third generation cephalosporins through extended-spectrum beta-lactamases (ESBLs) is the most frequently found antibiotic resistance of medical importance[1]. Unrecognized, infections with ESBL producing bacteria pose a serious threat, as they are associated with high morbidity and mortality rates[2]. *Escherichia coli* and *Klebsiella pneumoniae* are reported among the main representatives of ESBL-producing bacteria. In the Netherlands, ESBL prevalence is approximately 10 percent among infected patients[3].

Extended-spectrum beta-lactamases are a group of beta-lactamases which can also hydrolyze 3rd generation cephalosporins. The detection of these ESBL-enzymes is currently provided indirectly by the results of standard susceptibility testing of cultured bacteria, followed by a phenotypic confirmation assay or a genetic test. Direct detection of the enzyme responsible would provide molecular information regarding the phenotype. Protein analysis by way of mass spectrometry has changed microbiological practice in recent years through the introduction of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) for species identification[4]. However, the inherent limitations of these instruments such as the limited dynamic range and resolution limit the general applicability to accurately detect the presence of ESBL, particularly in identifying the nature of the underlying enzyme.

Peptide analysis by bottom-up proteomics is commonly used to directly identify proteins and can be used for in-depth proteomic characterisation of resistant bacteria, often using multi-dimensional protein and/or protein fractionation techniques[5-7]. However, straight analysis of proteolytic digests of total cellular protein extracts also allows to directly identify resistance related proteins such as beta-lactamases[8,9]. This leads to shorter analysis times compared to comprehensive proteome studies while maintaining the inherent specificity of directly identifying the protein of interest. Previously we developed such a proteomic platform for the direct detection of OXA-48 and KPC carbapenemases in bacterial cultures of clinical isolates[10-12].

A significant reduction in analysis time would be achieved when bacterial beta-lactamases could be directly analysed in positive blood cultures. Therefore, the aim of this study was to develop a LC-MS/MS based bottom-up proteomics workflow to identify ESBL-producing *E. coli* and *K. pneumoniae* directly in blood cultures and to test the performance of this workflow in a proof-of-principle study using clinical blood culture samples collected in a prospective study.

Materials and Methods

Design of study

This study was designed to evaluate the use of proteomic analysis by LC-MS/MS for the detection of extended-spectrum beta lactamases directly from positive blood culture bottles that grow *E. coli* or *K. pneumoniae*, during a prospective study. Two academic centres participated in the study: the Erasmus University Medical Center in Rotterdam and the Leiden University Medical Center in Leiden. During a period of 4 months (July-October 2015), all positive blood cultures with *K. pneumoniae* or *E. coli*, were included in the study.

Comparison of sample preparation methods

Two methods were evaluated for the analysis of bacterial proteins in blood cultures, serum separator tubes and a differential lysis protocol using saponin. For this purpose, negative blood culture bottles were spiked with different amounts of liquid broth culture of *E. coli* BL21(DE3) pLysS to mimic different bacterial densities in positive blood cultures.

Serum separator tubes feature a gel through which red blood cells can migrate while bacteria are pelleted on top of the gel. Four mL from a spiked blood culture bottle was applied to the tubes (Becton Dickinson, Breda, The Netherlands) and the mixture was centrifuged at 6000g for 10 min. Serum was removed and the pellet was washed twice with 1 mL of phosphate buffered saline (PBS) followed by a 5 min centrifugation at 6000 x *g*. The bacterial pellet on top of the gel was resuspended in 100 µL PBS and transferred to an Eppendorf tube. The gel was rinsed with 100 µL PBS another three times to recover any residual bacteria and this was added to the vial. The resulting bacterial suspension was centrifuged at 10.000 g for 1 minute and the supernatant was removed. 100 µL of 50% trifluoroethanol (TFE) solution was added for protein extraction and solubilisation. This suspension was sonicated in an ultrasound water bath for 2 min. Suspensions were heated to 60 °C for an hour. The resulting lysates were then subjected to protein digestion.

For the saponin protocol, 4 mL of the spiked blood culture bottle was mixed with 1 mL of a saponin (Sigma-Aldrich, Zwijndrecht, The Netherlands) stock solution (5% w/v, final concentration 1% w/v). The mixture was vortexed, incubated at room temperature for 5 min and centrifuged at 6000g for 10 min. The cell pellet was washed three times with 1 mL PBS and centrifuged at 10.000 g for 1 minute and, following the final centrifugation step, re-suspended in 100 µL 50% TFE solution. The suspension was sonicated using an ultrasound water bath for 2 min. Suspensions were heated at 60 °C for an hour. The resulting lysates were stored at -80 °C until further analysis.

Blood culture and species identification.

Blood cultures were drawn as part of normal clinical routine. 8-10 mL of blood was used per bottle (Bactec Plus Aerobic and Bactec Plus Anaerobic, Becton Dickinson, Breda, The Netherlands) for blood culturing (Bactec FX, Becton Dickinson, Breda, The Netherlands). Bacterial species in positive cultures were identified directly from 1 mL blood culture by MALDI-TOF MS analysis (Microflex, Bruker Daltonics, Bremen, Germany) according to an in-house developed protocol adapted from literature[13]. All positive flagged blood cultures were stored at 4 °C and processed within 48 hours using the saponin protocol described above.

Susceptibility testing

Susceptibility testing was performed with VITEK 2 (bioMérieux, Marcy l'Etoile, France). The presence of ESBLs was performed using the combination disk diffusion tests (Rosco Diagnostica A/S, Taastrup, Denmark) according to the Dutch guideline[14]. Disks used were ceftazidime (30 µg), ceftazidime + clavulanate (30+10 µg), cefotaxime (30 µg) and cefotaxime+clavulanate (30+10 µg). ESBL production was considered positive if the zone diameters around one or both of the combination disks was ≥ 5 mm compared to the corresponding antibiotic-only disk. E-tests for ceftazidime, cefotaxime and meropenem were performed on Mueller Hinton E agar media according to the recommendations of EUCAST (http://www.eucast.org/clinical_breakpoints/).

In–solution protein digestion

Stored lysates (at -80 ˚C) were thawed for further processing for bottom-up proteomics. Reduction was performed with dithiothreitol (DTT, final concentration 2.5 mM in 25 mM ammonium bicarbonate) at 60 °C for 15 min. Alkylation was performed in the dark with iodoacetamide (final concentration 5.5 mM in 25 mM ammonium bicarbonate) for 15 min. Following alkylation the samples were digested overnight using sequencing grade modified trypsin (12.5 ng/ml, Promega, Leiden, The Netherlands). The next day the resulting digests were lyophilized and reconstituted in 0.5% trifluoroacetic acid (TFA) for pre-column trapping during LC-MS/MS analysis.

Molecular characterisation

All ESBL positive isolates (n=22) were analyzed for the presence of beta-lactamase genes. An in-house real-time multiplex $bla_{CTX\text{-}M}$ PCR was used for analysis of the specific CTX-M groups. For primer design, an alignment of the available bla_{SHV} gene sequences from GenBank® was made using the AlignX program (Vector NTI Advance 11, Invitrogen). Primers and probes were developed in-house using Beacon Designer (Premier Biosoft, Palo Alto, U.S.A.). Subsequently, beta-lactamase gene bla_{SHV} was amplified using PCR and further investigated by nucleotide sequence analysis[15]. All primers and probes used in this study are listed in Table 1. These molecular assays have been developed and internally validated at the LUMC and are used in daily routine.

Table 1: Comparison of the number of protein identifications using two different sample preparation protocols for LC–MS/MS analysis of bacterial protein extract from blood cultures.

Samples were spiked with 3.0 10^7 or 3.0 10^8 CFU obtained from liquid broth culture. Saponin: differential lysis protocol. SST: Serum separator tube protocol. Reference reflects a samples directly taken from the O/N bacterial culture.

Sample	Bacterial proteins	Human proteins
Reference	566	16
Saponin 3.0 10^7 CFU	196	165
Saponin 3.0 10^8 CFU	477	135
SST 3.0 10^7 CFU	199	145
SST 3.0 10^8 CFU	288	82

LC–MS/MS analysis and data processing

Peptide mixtures were analysed using nano reversed-phase liquid-chromatography coupled to tandem mass spectrometry (nano LC-MS/MS). The nano-LC system (Ultimate 3000 RSLCnano, Dionex) combines a 2 cm Acclaim PepMap 100 guard column with an Acclaim PepMap RSLC column (C18, 75 µm x 50cm with 2 µm particles). A multi-step gradient going from 5 to 55% B in 180 min was used (solvent A being 0.1% formic acid in water and solvent B 0.1% formic acid in 80% acetonitrile) at a rate of 300 nL/min. Mass spectrometry analysis was carried out on a maXis Impact UHR-TOF-MS (Bruker Daltonics) in data dependent MS/MS mode, with precursors ranging from *m/z* 300-1200. After MS/MS analysis precursors were excluded from selection dynamically for one minute.

Raw data were converted to Mascot Generic Files (MGF) and analysed by database searching using the Mascot algorithm (Mascot 2.5.1, Matrix Science, London, UK) using Mascot Daemon 2.5.1. To ensure a comprehensive search of all beta-lactamases, a custom database was prepared. This database consists of *in-silico* translated reference genomes for *K. pneumoniae* (http://www.ncbi.nlm.nih.gov/genome/815?genome_assembly_id=168877) and *E. coli* (http://www.ncbi.nlm.nih.gov/genome/167?genome_assembly_id=161521), supplemented with a comprehensive list of beta-lactamases continued from the former Lahey database (December 2015, ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele-prot.fa). Searches were carried out with the following parameters: precursor mass tolerance was 0.05 Da, MS/MS tolerance 0.8 Da. Carbamidomethylcysteine was set as a fixed modification, with methionine oxidation as variable modification. Trypsin was designated as enzyme with a maximum allowed number of missed cleavages of two. The False Discovery Rate (FDR) was set at 0.01 at the peptide level based on decoy database searches.

Results

Optimization of sample extraction

Two different sample preparation protocols were compared with respect to the overall number of protein identifications and the ease of use of the method. For this purpose, we used negative blood culture bottles spiked with different amounts of liquid broth culture of *E. coli* BL21(DE3) pLysS cells to mimic different bacterial densities in positive blood cultures. The number of successful protein identifications was determined at $3.0x10^7$ and $3.0x10^8$ CFU using a sample preparation by serum separator tubes and by differential lysis protocol using saponin. As a reference, the protocols were also applied to the bacterial suspensions used for inoculation of the blood cultures, using $1.0x10^7$ CFU. Results of all analyses were searched independently against the bacterial database and, for human proteins, against the human database. Table 2 summarises the results for the proteomic comparisons of both protocols. The sample containing $3.0x10^8$ CFU performed better in the saponin protocol. Since this procedure is less laborious we treated all subsequent positive blood cultures by the saponin protocol. A number of variables of the saponin lysis protocol were tested including centrifugation speed and duration, saponin concentration and number of washing steps. No significant improvements were made and the protocol therefore remained unchanged.

Table 2: Collection of positive blood cultures.

Samples were collected in two university medical centers in the Netherlands: the Erasmus MC in Rotterdam and the Leiden University Medical Center (LUMC) in Leiden. Presence of ESBLs was determined using phenotypical susceptibility testing.

Origin	Positive blood cultures					
	E. coli	no. ESBL+ (%)	*K. pneu.*	no. ESBL+ (%)	Total	no. ESBL+ (%)
ErasmusMC	57	8 (14.0)	22	3 (11.1)	79	11 (13.9)
LUMC	68	10 (14.7)	23	1 (4.3)	91	11 (12.1)
Sum	125	18 (14.4)	45	4 (8.9)	170	22 (12.9)

Blood culture collection and susceptibility testing

During a period of four months, positive blood cultures with *E. coli* or *K. pneumoniae* were collected prospectively (Table 3). In total, 170 positive blood cultures were collected. Of these, 125 (73.5%) contained *E. coli* and 45 (26.5%) *K. pneumoniae.* Following susceptibility testing of cultured isolates, 22 isolates (12.9%, 18 *E. coli* and 4 *K. pneumoniae*) were confirmed as ESBL-positive with the combination disk diffusion test.

Table 3: Resistance profile and LC–MS/MS–based ESBL identification in ESBL positive blood cultures.

Culture	*Origin*	*Species*	*Resistance profile*			*β-lactamase detection*	
			MIC (mg/L)			LC-MS/MS	PCR
			CTX	CZD	Mero		
1	LUMC	*E. coli*	>32	8	0.023	Group 1 CTX-M	Gr 1 CTX-M
2	LUMC	*E. coli*	>32	24	0.032	Group 1 CTX-M	Gr 1 CTX-M; TEM
3	LUMC	*E. coli*	>32	3	0.023	Group 9 CTX-M; TEM	Gr 9 CTX-M; TEM
4	LUMC	*E. coli*	>32	3	0.023	Group 9 CTX-M	Gr 9 CTX-M; TEM
5	LUMC	*E. coli*	>32	8	0.012	Group 1 CTX-M	Gr 1 CTX-M
6	LUMC	*K. pneumoniae*	>32	32	0.032	Group 1 CTX-M	Gr 1 CTX-M; SHV-1; TEM
7	LUMC	*E. coli*	>32	1.5	0.023	Group 1 CTX-M	Gr 1 CTX-M
8	LUMC	*E. coli*	>32	4	0.023	Group 1 CTX-M	Gr 1 CTX-M; TEM
9	LUMC	*E. coli*	>32	6	0.023	Group 1 CTX-M	Gr 1 CTX-M
10	LUMC	*E. coli*	>32	2	0.023	Group 1 CTX-M	Gr 1 CTX-M
11	LUMC	*E. coli*	>32	6	0.023	Group 1 CTX-M; TEM	Gr 1 CTX-M; TEM
12	Erasmus MC	*E. coli*	>32	32	0.023	Group 1 CTX-M	Gr 1 CTX-M; TEM
13	Erasmus MC	*E. coli*	8	0.25	0.023	Group 9 CTX-M	Gr 9 CTX-M
14	Erasmus MC	*E. coli*	>32	6	0.023	Group 1 CTX-M; TEM	Gr 1 CTX-M; TEM
15	Erasmus MC	*K. pneumoniae*	8	32	0.032	SHV*	SHV-12; TEM
16	Erasmus MC	*E. coli*	>32	0.75	0.012	Group 9 CTX-M	Gr 9 CTX-M; TEM
17	Erasmus MC	*K. pneumoniae*	>32	8	0.032	Group 1 CTX-M	Gr 1 CTX-M; SHV-11; TEM
18	Erasmus MC	*E. coli*	>32	12	0.032	Group 1 CTX-M; TEM	Gr 1 CTX-M; TEM
19	Erasmus MC	*K. pneumoniae*	>32	48	0.094	Group 1 CTX-M	Gr 1 CTX-M; SHV-1
20	Erasmus MC	*E. coli*	>32	8	0.023	Group 1 CTX-M	Gr 1 CTX-M; TEM
21	Erasmus MC	*E. coli*	>32	8	0.023	Group 1 CTX-M	Gr 1 CTX-M
22	Erasmus MC	*E. coli*	>32	48	0.023	Group 1 CTX-M	Gr 1 CTX-M

MIC (mg/L) values were determined using E-tests. CTX: cefotaxime, CZD: ceftazidime, Mero: meropenem.
*: The MS/MS data was inconclusive about the positive identification of this SHV as an ESBL because the single peptide necessary to discriminate between an ESBL and non-ESBL was not identified. The sequencing of the PCR product confirmed that this was an ESBL. See text for further explanation.

Results from bottom–up proteomics analysis

All 22 blood cultures with ESBL positive isolates were selected for proteomic analysis, as well as 44 randomly selected ESBL negative blood cultures. Preparation of the 66 blood cultures for LC-MS/MS analysis was performed blind with regards to the results of the phenotypic testing. Following LC-MS/MS analysis, the resulting spectra were searched against the in-house generated database (see materials and methods) featuring a comprehensive list of beta-lactamases as well as *K. pneumoniae* and *E. coli* proteomes. In a typical analysis of one positive blood culture bottle, 400-800 bacterial proteins were identified. Table 4 summarises the results for the phenotypically ESBL positive blood cultures (n=22). In all analyses results obtained by MS, the detected β-lactamase was always in the top 10% of the total number of identified bacterial proteins in a sample, sorted by identification score. In 21 out of 22 of the ESBL positive isolates a cefotaximase (CTX-M) was identified. Protein sequence coverage based on identified peptides varied from 38% to 88%. This coverage allows for the mapping of the identified cefotaximases into one of six established lineages[16], named after their archetypical enzymes. In our collection, only members of groups CTX-M-1 and CTX-M-9 were found. In one *K. pneumoniae* isolate (Table 4, number 15) no cefotaximase was found. A SHV type beta-lactamase was identified with 33% coverage (Figure 1). Like with the cefotaximases, this protein was a top 10% identification among all bacterial proteins identified. From 148 ESBL-negative *K. pneumoniae* or *E. coli* positive blood cultures, 44 were randomly selected and also analysed by LC-MS/MS. In none of these samples, extended-spectrum beta-lactamases were found.

Coverage of SHV-1 sequence:

MRYIRLCIIS	LLATLPLAVH	ASPQPLEQIK	**LSESQLSGRV**	**GMIEMDLASG**
RTLTAWRADE	**R**FPMMSTFKV	VLCGAVLAR**V**	**DAGDEQLER**K	IHYR**QQDLVD**
YSPVSEKHLA	**DGMTVGER**CA	AAITMSDNSA	ANLLLATVGG	**PAGLTAFLRQ**
IGDNVTRLDR	WETELNEALP	GDAR**DTTTPA**	**SMAATLR**KLL	TSQRLSARSQ
RQLLQWMVDD	RVAGPLIRSV	LPAGWFIADK	TGA<u>G</u>ERGAR**G**	**IVALLGPNNK**
AERIVVIYLR	**DTPASMAER**N	QQIAGIGAAL	IEHWQR	

Figure 1: Coverage of SHV-1 sequence. Identified peptides in LC-MS/MS analysis are highlighted in red when they matched to the sequence of SHV-1 beta-lactamase. The glycine at Ambler position 238 (underlined) is specific for SHV-1 sequence, while SHV-2 type extended-spectrum beta-lactamases have a serine in this position. This peptide was not observed in LC-MS/MS analysis making it not possible to distinguish between the beta-lactamase types.

Molecular characterisation of ESBL positive isolates

To confirm the identity of the ESBLs identified with LC-MS/MS based proteomics, molecular characterisation of all phenotypically ESBL positive isolates was performed (Table 4). All CTX-M identifications were verified with PCR. In isolate 15, the LC-MS/MS identified a SHV-enzyme which was confirmed by PCR as an ESBL, namely SHV-12. Three non-ESBL SHV beta-lactamases were identified by PCR in the *K. pneumoniae* isolates.

Discussion

In this study we developed a novel proteomic workflow for the direct identification of ESBLs in positive blood culture bottles. To evaluate the performance of our approach, a proof-of-principle prospective study was performed in two academic hospitals. In 22 positive blood cultures with phenotypically ESBL producing *E. coli* or *K. pneumoniae*, we identified 21 isolates containing a CTX-M and one isolate containing a SHV beta-lactamase, although the latter could not unambiguously be identified because the single peptide necessary to discriminate between an ESBL and non-ESBL was not identified. In the set of positive blood cultures with ESBL-negative *E. coli* or *K. pneumoniae*, no ESBLs were identified by LC-MS/MS analysis. This demonstrates a 95% sensitivity and 100% specificity of the workflow to directly identify these beta-lactamases in positive blood cultures.

Of 170 positive blood cultures collected in two academic hospitals, 22 (12.9%) contained ESBL producing bacteria belonging to *E. coli or K. pneumoniae*. This percentage is higher than previously described in The Netherlands[3], with cefotaxime/ceftriaxone resistances reported to be 5% and 7% for *E. coli* and *K. pneumoniae* respectively. However, these data were based on a larger number of laboratories, including laboratories serving non-university hospitals and general practitioners. All collected samples in our study showed full meropenem susceptibility, in agreement with the low prevalence rate of carbapenemase producing Gram-negatives in the Netherlands[3].

In the proteomic analysis of the clinical isolates, 21 out of 22 (95%) of the phenotypically ESBL positive isolates contained a CTX-M ESBL. Cefotaximases are the most widespread ESBLs and a high representation in this study was expected[16,17]. In our study, the CTX-M enzymes belonged to group 1 (17 out of 21, 81%) and group 9 (4 out of 21, 19%). This is in accordance with other reports[18]. Specifically, CTX-M-15 (group 1) and CTX-M-14 (group 9) are among the most prevalent enzymes[19]. Notably, in our collection there was no relation between MIC and ESBL type, especially not for ceftazidime.

Full sequence coverage is necessary to pinpoint a protein identification to a specific ESBL but in complex samples with the use of only one proteolytic enzyme, this is not feasible. Obviously, peptide fractionation or additional experiments with another proteolytic enzyme could improve the specificity of the identification. However, we opted for a simple sample preparation protocol, which is mostly constrained time-wise by the proteomic digestion step. In our approach, the sequence coverage among ESBLs in phenotypically positive isolates ranged between 38% and 88%. This coverage is in-depth enough to classify the enzymes into phylogenetic groups, such as with the cefotaximases, but single variants cannot be distinguished using this method. This is important in distinguishing beta-lactamases that have reported broad and extended-spectrum activities, based on small permutations.

For example, one blood culture sample contained a SHV beta-lactamase. The sequence coverage obtained by LC-MS/MS analysis was not sufficient to distinguish between a broad and extended-spectrum beta-lactamase. The amino acid at position 238 is instrumental in cephalosporin resistance in SHV variants and the tryptic peptide covering this amino acid is therefore necessary for the unambiguous assignment of the ESBL status[20]. The corresponding tryptic peptides of the SHV-1 (broad spectrum) and SHV-2 (extended-spectrum) are TGAGER and TGASER, respectively. While the double charged state would be within the mass range of the mass spectrometer, sensitivity in this low mass range is not optimal and short peptides can also be difficult to retain and separate in liquid chromatography. A more targeted approach might be more suitable for such specific peptides[21]. Importantly, the LC-MS/MS identified SHV beta-lactamase in isolate 15 was confirmed to be an ESBL (SHV-12) by our PCR and sequence analysis. The PCR analyses revealed three additional SHV beta-lactamases which were not identified in the proteomics analysis. Sequence analysis demonstrated that the three additional SHV beta-lactamases were non-ESBLs (SHV-1, SHV-11) and could have been missed in our proteomic analysis due to lower abundance as compared to the ESBL-SHV. Therefore, as it stands now, identifying a SHV with high expression combined with the phenotypical results indicates an ESBL-SHV, but our proteomic data was not sufficient to unambiguously draw this conclusion.

In this proof-of-principle study, ESBLs from the CTX-M group were easy to identify. PCR-based methods have been successfully applied for the identification of ESBLs in blood cultures[22,23] and in our study, the CTX-M PCR results fully correlated with the proteomics results. The aim of our prospective study was to demonstrate the applicability in normal routine, and therefore we detected mainly CTX-M ESBL. Larger clinical sample cohorts and spiking experiments with other ESBL/carbapenemase producing bacteria in negative blood cultures are necessary to demonstrate the general applicability of our approach. Based on the results of our previous study, this workflow should also be suitable for the detection of OXA-48 and KPC beta-lactamases directly in blood cultures[11]. The sample preparation is highly similar and overall proteome and protein coverage was significantly higher using this nanoLC platform. Moreover, the mass spectrometric analysis part of our workflow can be easily exchanged for other high-end mass spectrometry analysers (such as Orbitraps) with even higher speed and sensitivities. As with all genetic methods, a positive identification does not guarantee protein expression. More sensitive proteomic analysis could therefore give some insight into our problem to detect the additional non-ESBL lactamases with our proteomics workflow.

Apart from genetic tests, there are alternative methods to detect the presence of ESBLs in blood culture bottles. Oviaño *et al*. monitored ESBL activity directly from blood cultures using MALDI-TOF MS by measuring antibiotic hydrolysis[24]. Reported sensitivity and specificity are high, suggesting that such an approach can be used as an alternative to traditional susceptibility testing. Moreover, hydrolysis based assays using reporter molecules are

mentioned in literature and available as commercial kits[25,26]. Even though hydrolysis based tests are useful, interpretation can be difficult in case of enzymes with a lower activity, and they provide no insight in the identity of the ESBL. In comparison with genetic and hydrolysis based methods, our workflow allows the direct identification of the enzyme responsible, providing molecular information about the phenotype.

Overall, in this proof-of-principle prospective study we demonstrate the direct identification of an ESBL in all blood cultures that contained bacteria positive for a CTX-M type ESBL. The method is specific enough to recognise specific groups of CTX-M ESBL. To improve on this proof-of-principle study in the future a number of aspects need further exploration. Among these, shortening the time-to-report and automation of the procedure are among the most critical[27,28]. With this in mind, the developed platform can be used in the future for the direct identification of expressed beta-lactamases in blood cultures which provides detailed insight into the antibiotic resistance mechanism.

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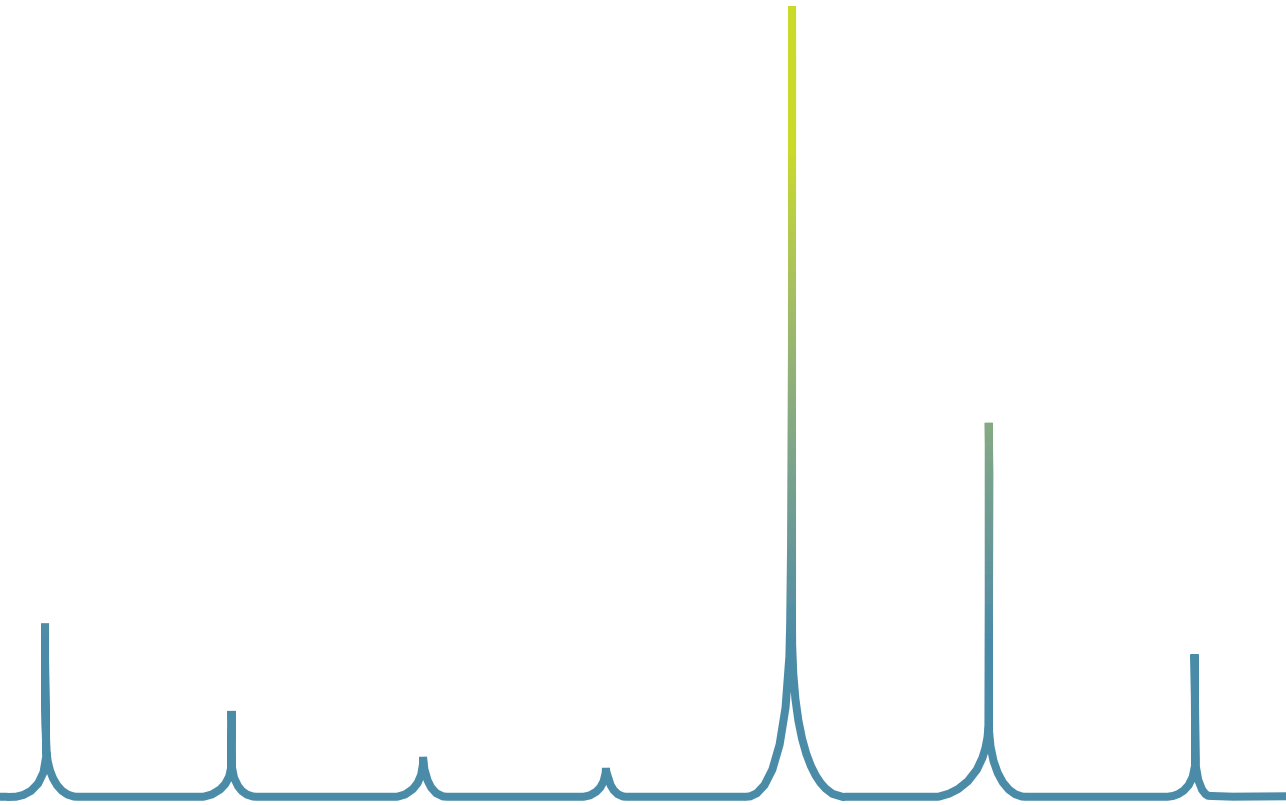

CHAPTER 4

Typing *Pseudomonas aeruginosa* isolates with ultrahigh resolution MALDI–FTICR mass spectrometry

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Abstract

The introduction of standardized matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) platforms in the medical microbiological practice has revolutionized the way microbial species identification is performed on a daily basis. To a large extent this is due to the ease of operation. Acquired spectra are compared to profiles obtained from cultured colonies present in a reference spectra database. It is fast, reliable and costs are low compared to previous diagnostic approaches. However, the low resolution and dynamic range of the MALDI-TOF profiles have shown limited applicability for the discrimination of different bacterial strains, as achieved with typing based on genetic markers. This is pivotal in cases where certain strains are associated with e.g. virulence or antibiotic resistance. Ultrahigh resolution MALDI-FTICR MS allows the measurement of small proteins at isotopic resolution and can be used to analyze complex mixtures with increased dynamic range and higher precision than MALDI-TOF MS, while still generating results in a similar timeframe.

Here we propose to use ultrahigh resolution 15T MALDI-FTICR MS to discriminate clinically relevant bacterial strains. We used a collection of well characterized *Pseudomonas aeruginosa* strains, featuring distinct antibiotic resistance profiles, and isolates obtained during hospital outbreaks. Following cluster analysis based on amplification fragment length polymorphism (AFLP), these strains were grouped into three different clusters. The same clusters were obtained using protein profiles generated by MALDI-FTICR MS. Subsequent intact protein analysis by ESI-CID-FTICR MS was applied to identify protein isoforms that contribute to the separation of the different clusters, illustrating the additional advantage of this analytical platform.

Introduction

The introduction and the development of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) strategies as a profiling tool for the analysis of bacterial peptides and proteins has revolutionized applications in clinical microbiology[1,2]. With the implementation of MALDI-TOF MS systems for *in-vitro* diagnostic applications, this technology has been introduced worldwide as a routine identification tool for characterizing bacteria and yeasts in clinical microbiology laboratories. The accurate identification of unknown microorganisms is achieved by comparing MALDI-TOF MS profiles obtained from cultured colonies to a database of reference spectra, without any *a priori* knowledge[3]. MALDI-TOF MS-based methods are high-end analytical tools that provide accurate data within a few min measurement time. These advantages, compared to conventional analysis[4-6] are the main drivers behind the success of MALDI-TOF MS and the establishment of the technique as part of controlling infectious diseases and outbreaks in hospitals[7-10].

One of the strongest points of the approach is the straightforward sample preparation and data acquisition protocol which does not require well-trained and experienced operators as required in other methods for identification of bacteria[11]. Bacterial colonies, cultured on solid media, are directly deposited onto a MALDI target plate and covered with a MALDI matrix solution (*e.g.* an α-cyano-4-hydroxycinnamic acid solution). To improve the quality of the spectra, a protein extraction procedure (*e.g.* using a formic acid solution) can be performed either before spotting or directly on the MALDI target plate. When bacteria are cultured on a support other than solid media, such asblood culture bottles, a more elaborate sample preparation is required to extract the bacterial proteins and generate the MALDI-TOF MS spectra[12,13]. Currently, MALDI-TOF MS is used to identify a variety of microorganisms from different sources including skin, faeces and body fluids[14].

In general, only high-abundant proteins such as ribosomal proteins are detected using direct MALDI-TOF MS profiling of bacterial extracts[15]. This usually limits the identification of microorganisms to a genus or species level. However, bacterial species can be genetically extremely heterogeneous and the typing of bacterial strains with specific phenotypic characteristics (*e.g.* associated with pathogenicity, antibiotic resistance and/or virulence) is, in many cases, equally important[16]. In case of an outbreak where a multidrug-resistant bacterium can quickly spread in the hospital, a prompt identification and localization of the source is required to stop transmission, and efficiently treat the infection. A few examples have shown that MALDI-TOF MS can also be used for typing bacterial strains[17-20] but, in general, this application is strongly limited by the low dynamic range and low resolution of MALDI-TOF MS instruments.

Genotyping is nowadays the most common strategy to discriminate bacterial strains. For this purpose different methods are available, each with its pros and cons[21]. In our medical center, amplified fragment length polymorphism (AFLP) is routinely applied on bacterial isolates when an outbreak with a specific strain is suspected[22,23]. Moreover, whole-genome sequencing provides complete information on genetic diversity of bacterial strains. This method is expensive however, and although continuous developments in technology keep reducing time of analysis, it is still time-consuming[24].

In 2003, Jones and co-workers pioneered in using MALDI-Fourier transform ion cyclotron resonance (FTICR) MS for analysis of bacterial proteins up to 10 kDa, resulting in higher mass accuracy and resolution but lower dynamic range than MALDI-TOF MS[25]. Since then, with further development of FTICR systems and availability of higher magnetic fields, the quality of protein profiles generated using MALDI-FTICR MS has drastically improved[26]. Recently we have shown full isotopic resolution of proteins up to about 17 kDa that were measured on a MALDI-FTICR MS system equipped with a 15T magnet[27]. Likewise, complex protein mixtures (*e.g.* human serum proteins) have been analyzed in more detail with a dynamic range, and mass measurement precision and accuracy that are superior to those obtained with MALDI-TOF MS[28-31].

The aim of this study is to use ultrahigh resolution MALDI-FTICR MS to type bacterial strains by profiling bacterial proteins in more detail than MALDI-TOF MS. To this end, different clinical isolates of *Pseudomonas aeruginosa*, including multidrug-resistant types, associated with hospital outbreaks[22] were analyzed using 15T MALDI-FTICR MS. Cluster analysis of the MALDI-FTICR MS data resulted in a similar classification as observed with AFLP. Intact protein analysis by collision-induced dissociation (CID) was subsequently performed by direct infusion ESI-FTICR MS/MS to identify proteoforms that differ between clusters, signifying the additional power of the analysis platform.

Materials and Methods

Bacterial isolates

Pseudomonas aeruginosa strains were obtained from the Department of Medical Microbiology at the Leiden University Medical Center (LUMC, Leiden, the Netherlands) and the Department of Medical Microbiology & Infectious Diseases at the Erasmus Medical Center (Rotterdam, the Netherlands) (see Table S1 in Supporting Information). Two of these bacterial strains (*i.e.* KML 01 and KML 04) were previously identified during the outbreak described by Knoester and co-workers[22].

Antimicrobial susceptibility testing was performed using the VITEK2® system (BioMérieux, Marcy-l'Étoile, France). Susceptibility results were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/).

Table 1. *P. Aeruginosa* strains used for this study

P. Aeruginosa Strain Nr.	Place of Collection
KML 01*	LUMC
KML 02	LUMC
KML 03	LUMC
KML 04*	LUMC
KML 05	LUMC
KML 06	LUMC
KML 07	LUMC
KML 08	LUMC
KML 09**	LUMC
KML 10	LUMC
KML 11	Erasmus MC
KML 12	Erasmus MC
KML 13	Erasmus MC
KML 14	Erasmus MC
KML 15	Erasmus MC
KML 16	Erasmus MC
KML 17	Erasmus MC
KML18	Erasmus MC

* Bacterial strains reported by Knoester et al. (29)

** No AFLP data available

AFLP analysis

Nucleic acids of *P. aeruginosa* were extracted using the Qiasymphony SP robot with the Total Nu celic Acid kit (Qiagen, Hilden, Germany). For AFLP typing, the DNA was digested using EcoRI and MseI restriction enzymes and subsequently the corresponding adaptors were ligated[23]. After amplification using labeled primers, the fluorescent amplified fragments were separated by the genetic analyzer ABI PRISM ® 3500xL (Applied Biosystems, Foster City, U.S.A.). AFLP patterns were analyzed using BioNumerics software, version 7.1 (Applied Maths, St.-Martens-Latem, Belgium). Similarity between normalized AFLP patterns (range 60 to 600 bp) was calculated with the Pearson product-moment correlation coefficient and UPGMA algorithms.

Bacterial protein extraction and MALDI spotting

P. aeruginosa clinical isolates and a reference *P. aeruginosa* strain (KML 10) were incubated on sheep blood agar (BioMérieux, Marcy-l'Étoile, France) overnight at 37 °C. Cultured colonies were sampled with an inoculation loop, transferred in an Eppendorf tube and suspended in 150 µL deionized water. Then, 450 µL of ethanol was added, vortexed and the suspension was centrifuged at 13,000 x *g* in a benchtop centrifuge for two min. The supernatant was removed and the pellet reconstituted in 25 µL of 70% formic acid. Subsequently, 25 µL of acetonitrile was added and mixed using a pipette. The suspension was then centrifuged again for 2 min. One µL of the supernatant was spotted and mixed with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) MALDI-matrix solution (saturated solution in 50% acetonitrile, 5% trifluoracetic acid) directly onto either a ground-steel MALDI target plate for MALDI-FTICR MS analysis or a polished steel MALDI target plate for MALDI-TOF MS Biotyper analysis.

The bacterial cultures were stored at 4°C and bacterial protein extraction was repeated from new colonies after 24 hours and 48 hours.

For LC-MS experiments, the bacterial proteins from the same *P. aeruginosa* strain were extracted in triplicate (*i.e.* from three different colonies). The bacterial protein extracts from these triplicates were mixed and concentrated to near dryness using a speed-vacuum system. Then, 25 µL of LC mobile phase A (see below) was added, vortexed and centrifuged for 5 min at 16,220 rpm (Mod. 5427 R; Eppendorf, Nijmegen, the Netherlands). Twenty µL of the supernatant was then transferred into a glass vial and 4 µL were injected into the LC system.

MALDI–TOF mass spectrometry (Biotyper)

All isolates were analyzed using a MALDI Biotyper system (BrukerDaltonics, Germany) and the Biotyper software 3.0. This software is used to compare a MALDI Biotyper spectrum from an unknown bacterium with a database of reference spectra. This comparison is based on pattern recognition and leads to a score value with the following interpretation: a score between 0.000 and 1.699 corresponds to a poor (unreliable) identification; a score between 1.700 and 1.999 results in a good genus identification; a score between 2.000 and 2.299 results in a secure genus identification and good species identification; a score between 2.300 and 3.000 corresponds to an excellent species identification.

For cluster analysis, raw spectra were pre-processed using the default method for mean spectrum profile (MSP) creation available in the Biotyper 3.0 software. The reference peak list of each MSP was pairwise matched to each other, the resulting matching scores were used to generate a MSP dendrogram. The distance level of the MSP dendrogram was normalized to a maximum value of 1.000.

MALDI– and ESI–FTICR mass spectrometry

Both MALDI- and ESI-FTICR MS experiments were performed on a Bruker 15T solariX XR FTICR mass spectrometer controlled by ftmsControl software and equipped with a CombiSource and a novel ParaCell (Bruker Daltonics,Bremen, Germany). A Bruker Smartbeam-II Laser System was used for irradiation at a frequency of 500 Hz using the "medium" predefined shot pattern. Ions were generated and measured as previously reported[32] with some modifications. Briefly, each MALDI-FTICR spectrum was generated from 200 laser shots and in the *m/z*-range from 3497.3 to 30,000 with 512K data points (*i.e.* transient length of 3.986 s). The ParaCell parameters were as follows: the DC bias RX0, TX180, RX180, and TX0 were 5.13, 5.20, 5.27, 5.20 V respectively; the trapping potentials were set at 4.5 V and 4.4 V; the excitation power and sweep step time 55 % and 13.5 µs. The transfer time of the ICR cell was 2.4 ms and the quadrupole mass filter was set at *m/z* 3,300.

Both LC-ESI-FTICR MS and direct infusion (DI) experiments were performed in the *m/z*-range from 306.80 to 3,000 with 1M data points. The ParaCell parameters were as follows: the DC bias RX0, TX180, RX180, and TX0 were 1.430, 1.500, 1.570, 1.500 V, respectively; the trapping potentials were set at 1.5 V and 1.5 V; the excitation power and sweep step time 22 % and 15 µs. The transfer time to the ICR cell was 1.1 ms. LC-ESI-FTICR MS experiments were performed using the quadrupole filter set at *m/z* 322, an accumulation time in the hexapole of 0.250 s and three averaged scans per retention time point. Direct infusion ESI-FTICR MS experiments were carried out at an infusion rate of 2 µL/min. Precursors ions were isolated through a quadrupole (Q), accumulated in the hexapole collision cell and fragmented by collision-induced dissociation (CID). Accumulation times and collision energies were optimized for each precursor ion. DataAnalysis Software 4.2 (Bruker Daltonics) was used for the visualization and interpretation of both MALDI- and ESI-(CID)-FTICR spectra.

Reversed–Phase Liquid Chromatography

An Ultimate3000 LC system (Thermo Scientific, Breda, The Netherlands) was coupled to the ESI source of the 15T SolariX XR FTICR MS system (Bruker Daltonics, Bremen, Germany). Four µL of the concentrated bacterial protein extract (*i.e.* from three replicate extractions) were injected onto a Luna RPC18 column (150 × 1 mm, 3 µm, Phenomenex, Utrecht, The Netherlands). Proteins were separated using a linear gradient starting from 0% B to 40% B in 30 min, then 100% B for 4 min and re-equilibration at 0% B for 11 min and a flow rate of 50 µL/min. The mobile phases consisted of 0.5% formic acid in 5% acetonitrile and 94.95% milliQ water for A and 0.5% formic acid in 94.95% acetonitrile and 5% milliQ water for B. Fractions were collected manually every 1 min in an Eppendorf tube. Each fraction was diluted with 50 µL of an aqueous solution of 0.05% formic and 50% acetonitrile solution prior to DI-ESI-FTICR MS experiments.

MALDI–FTICR–MS spectra processing

Each bacterial protein extract from the 18 *P. Aeruginosa* strains was spotted in duplicate and measured by MALDI-FTICR MS. All spectra were visually inspected and for each duplicate the spectrum with the highest signal intensity was selected for further processing. An isotope cluster was identified when at least three isotopic peaks were detected with a S/N higher than 10. For each detected isotope cluster in the spectra the *m/z*-value of the three most abundant isotopic peaks was included in a so-called reference file. For each isotope peak a certain *m/z*-window was also defined. Using the in-house developed Xtractor software[33], all the intensities of the data points within the defined *m/z*-windows were summed and the resulting peak intensity was assigned to the corresponding *m/z*-value. In this way, all isotope clusters were accurately quantified even when partial overlaps occurred. The signal intensity of the three most abundant isotopic peaks were then summed and assigned to the most intense isotopic peak of the corresponding peptide or protein. The resulting list of *m/z*-values and peak intensities was used for further statistical analysis.

Data analysis and statistics

Peak intensities were median-standardized and adjusted for the interquartile range prior to subsequent analysis. Hierarchical linkage clustering was used to generate dendrograms using the Euclidean distance measure and average linkage. Classification between groups was verified using the Random Forest classifier. Classifications were generated using leave-one-out cross-validation and verified using the out-of-bag classifications provided by the classifier. Importance measures generated by the Random Forest were saved for subsequent inspection and identification of important discriminatory proteins.

Results and discussion

Rapid identification and typing of bacteria is pivotal for infection management and treatment control[34]. Our aim was to develop a new MS-based profiling method for typing of bacterial strains using ultrahigh resolution MALDI-FTICR MS which provides superior performance as compared to MALDI-TOF MS. For this purpose, we selected a set of well-defined clinical isolates of *P. aeruginosa*, an opportunistic pathogenic Gram-negative bacterium, commonly causing disease in immunocompromised patients. Moreover, this bacterium is known as a nosocomial pathogen, with hospital equipment acting as a possible reservoir, sometimes leading to an outbreak[35-38]. The collection of *P. aeruginosa* isolates was inititiated after an outbreak which occurred at the intensive care unit of the LUMC[22]. The collection used in this study features two strains from this outbreak (KML 01 and KML 04) as well as additional strains collected at later time points at the LUMC and Erasmus MC. An additional reference *P. aeruginosa* strain was also included.

First of all, the isolates were analyzed using MALDI-TOF MS on the Biotyper platform. For such standard analyses, ions are measured between *m/z* 2000-20,000 in low resolution spectra in which ions are detected as relatively broad peaks (Figure 1A and C). The number of peaks with a signal-to-noise (S/N) above 3 ranged between 71 (lowest) to 100 (highest) in the best spectrum. Moreover, as confirmed by isotopic resolution measurement by MALDI-FTICR MS, the majority of peaks in the *m/z*-range from 2,000 to 5,000 were doubly charged ions of the major species observed at higher *m/z*-values. Despite these limitations, all different strains were correctly identified as *P. aeruginosa* with high identification scores ranging from 2.231 to 2.477. When different reference strains are present in the database, the MALDI-TOF MS spectrum of an identified bacterium is matched against these reference spectra and an identification score is given. For example, Oumeraci and co-workers[20] mapped 5547 *P. aeruginosa* isolates to five individual Biotyper database entries. In our study, the *P. aeruginosa* strains were matched against the following reference database entries (*i.e.* different strains): ATCC 27853 THL, DSM 50071T_QC DSM, DSM 1117 DSM, DSM 50071T HAM, 19955_1 CHB, LMG 8029 LMG, 8147_2 CHB, A07_08_Pudu FLR and DSM 1128 DSM. For each analyzed *P. aeruginosa* strain an identification score higher than 2.000 was obtained for more than one reference strain. For example, a good match to all nine *P. aeruginosa* entries was obtained for the strain KML 05 with identification scores ranging from 2.043 to 2.352. The ranking of these identifications was not consistent between duplicate measurements demonstrating that an unambiguous identification of the most representative reference strain was not possible using MALDI-TOF MS analysis.

The bacterial protein extracts from the same *P. aeruginosa* strains were subsequently measured by ultrahigh resolution MALDI-FTICR MS. Peptides and proteins were detected in the *m/z*-range from 3,497 to about 15,000. The high resolving power is reflected by the different protein signals being isotopically resolved in MALDI-FTICR MS measurements whereas these are indistinguishable in MALDI-TOF MS analysis (Figure 1B and D). Moreover, the S/N is enhanced in the FTICR MS spectra. When the number of distinguishable features between MALDI-TOF MS and MALDI-FTICR MS analysis of bacterial extracts are compared (Figure 1C and D), an improved data quality is noticed with MALDI-FTICR. In total, 279 unique isotope clusters were observed in the 18 MALDI-FTICR MS spectra obtained from the different *P. aeruginosa* strains. These include doubly charged ions which were also observed, but to a higher extent, in the corresponding MALDI-TOF MS spectra. A database search for bacterial species identification using MALDI-FTICR MS spectra was not possible since these spectra are not compatible with the MALDI Biotyper software. Thus, the spectra were further processed (see Materials and Methods) to perform clustering analysis.

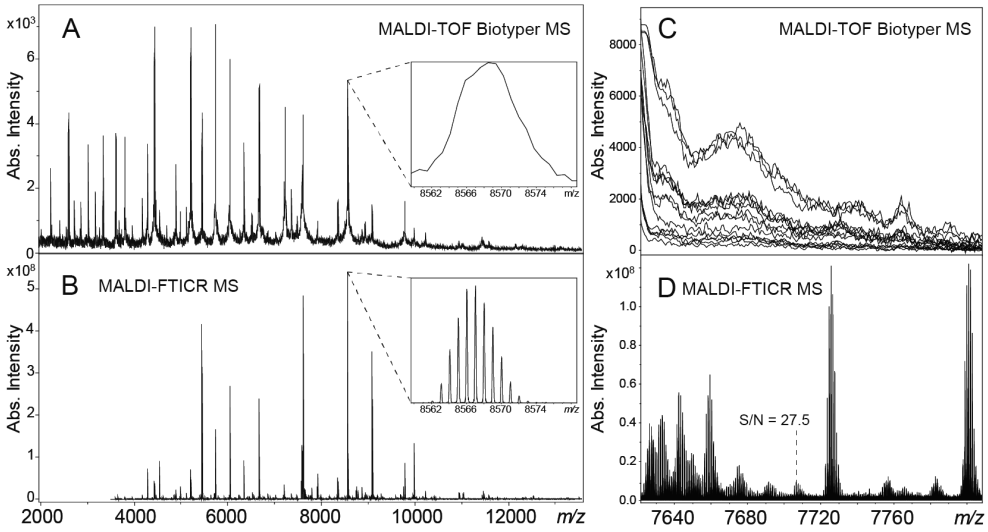

Figure 1: MALDI–TOF and MALDI–FTICR bacterial protein profiles of *P. aeruginosa.*

A) Typical low resolution MALDI-TOF spectrum obtained in the clinical laboratory using a Biotyper system; B) Ultrahigh resolution MALDI-FTICR spectrum acquired using a 15T solariX XR FTICR MS system. Insets: enlargements illustrating the difference in resolving power between the spectra generated by MALDI-TOF and MALDI-FTICR MS. C and D: Enlargement of overlaid spectra of multiple isolates, illustrating the higher dynamic range and resolving power provided by MALDI-FTICR MS.

We tested the possibility of using MALDI-TOF and MALDI-FTICR MS data for differentiation of the different *P. aeruginosa* isolates. To benchmark the genetic diversity, the collection was first characterized using AFLP (Figure 2A). Three different clusters appeared upon using the AFLP method. It has been shown previously that isolates in cluster 1 are resistant to ciprofloxacin, while strains in cluster 2 are susceptible[22]. The third cluster has previously not been identified. The non-outbreak related reference strain (KML 10) was positioned most closely to cluster 1.

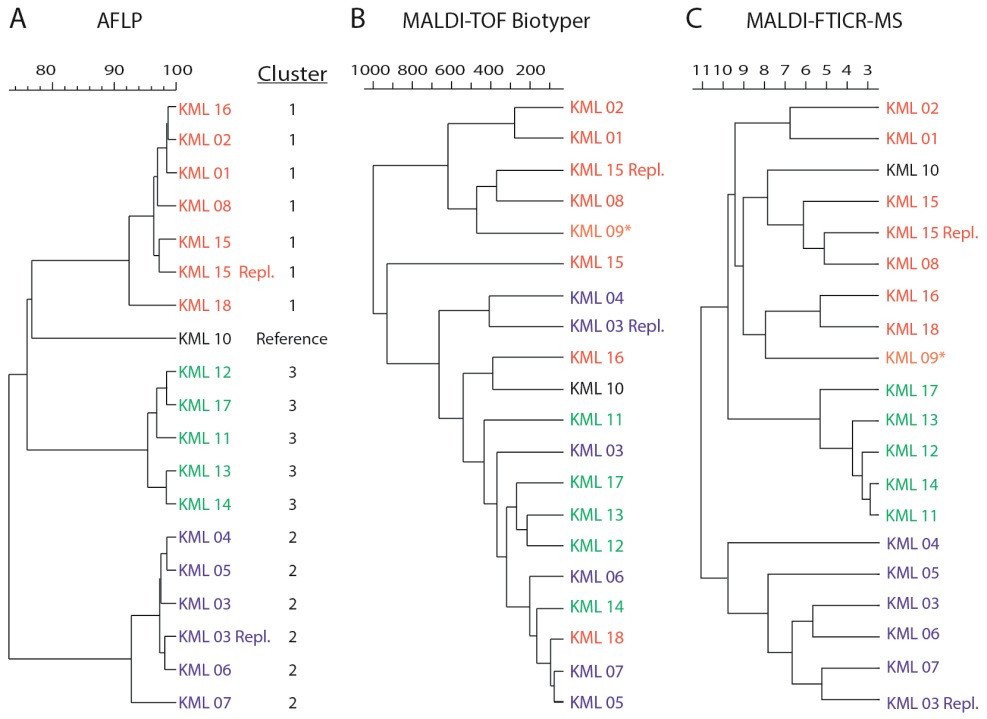

Figure 2: Side–by–side comparison of clustering dendrograms based on AFLP, MALDI–TOF Biotyper and MALDI–FTICR MS profiling of *P. aeruginosa* clinical isolates.

A) Using AFLP genotyping, three distinct clusters were identified. B) Partial and inaccurate clustering was obtained from MALDI-TOF MS data. C) Cluster analysis based on MALDI-FTICR MS analysis resulted in the same three groups identified using AFLP data. * No AFLP data available. KML 10 is a reference *P. aeruginosa* strain.

Cluster analysis based on the MALDI-TOF MS spectra revealed some clustering of strains belonging to cluster 1 but overall no clear separation as achieved with AFLP was obtained (Figure 2B). Importantly, the improved quality of bacterial protein profiles obtained using ultrahigh resolution MALDI-FTICR MS, such as the higher dynamic range, allowing the use of peak intensity values to classify the different *P. aeruginosa* strains, revealed a clustering of the *P. aeruginosa* isolates into the same three groups as obtained using the AFLP data (compare Figure 2A and C). The main advantage of typing bacteria based on MALDI-FTICR MS data is that this analysis can be performed at similar speed as the identification using MALDI-TOF MS. This means that in principle the typing of a novel isolate, or the classification of a collection of clinical isolates in an outbreak scenario, can be performed using MALDI-FTICR MS the same day of the identification of bacteria using MALDI-TOF MS. Successful and reliable same day typing can be an invaluable tool for infection management. For example, in our set discrimination between cluster 1 and 2 is essential because of the difference in susceptibility towards ciprofloxacin.

Within the clusters, some differences between AFLP and MALDI-FTICR MS were observed, which probably derive from two main factors: 1) AFLP and MALDI-FTICR MS provide limited and different information on genetic variation (*i.e.* AFLP at the DNA level and MALDI-FTICR MS at the protein level); 2) the relative abundance of proteins as observed with MALDI-FTICR MS is affected by growing conditions. In fact, it is well known that colonies from replicate cultures or even from the same culture plate can be very heterogeneous. The effect of this factor can be seen on the similarity between replicate cultures of the strains KML 03 and KML 15. Moreover, changes in similarity were observed in replicate measurements performed on three different days (Figure S1 in Supporting Information); importantly, however, the overall clustering into three groups remained the same. Further studies are needed to evaluate whether and how the changes in protein expression between colonies or growing conditions would affect a clustering analysis based on both MALDI-FTICR MS data and a pattern recognition method (*i.e.* without use of peak intensities).

As mentioned above, the identification of bacteria based on MALDI-FTICR MS data was not possible because no database of MALDI-FTICR reference spectra is available. The implementation of such a database would probably allow a more accurate and reliable identification of bacterial species. However, considering the cost of such expensive technology, it is more realistic to develop the platform towards the typing of bacteria (*e.g.* during an outbreak) in parallel to the validated MALDI-TOF based species identification which is already implemented in the clinic.

Top–down mass spectrometry identification of proteins important for the classification of *P. aeruginosa* strains

The discriminative power of each protein of the different *P. aeruginosa* strains can be visualized by plotting their relative importance in the classification (Figure 3A). Proteins with a discriminative value higher than 4 were considered as major contributors. Then, a search was performed to trace these discriminating proteins in the corresponding LC-ESI-FTICR MS data. A protein observed at *m/z* 7354.0032 in the MALDI-FTICR MS spectra (Figure 3 A, blue dot and inset), was found in the LC-ESI-FTICR MS data from strain KML 18. The LC fraction containing this protein was further analyzed by direct infusion ESI-FTICR-MS/MS aiming for top-down identification. In the ESI-FTICR MS spectrum the protein was detected at *m/z* 1051.4163 $[M+7H]^{7+}$ and *m/z* 920.1132 $[M+8H]^{8+}$. The latter ion was selected for CID fragmentation and the resulting spectra were manually annotated, which resulted in a sequence tag TATTQA(I/L)Q(I/L)(I/L)QN. Following BLAST searches in the UniProt database, the sequence tag TATTQALQILQN fully matched with the C-terminal region of an uncharacterized protein from *P. aeruginosa* (UniProt, entry A0A0M2DCU1). Moreover, the ion at *m/z* 433.2774, from where the sequence tag started, corresponds to the y_4 fragment containing the four C-terminal amino acids (TGKK, Figure 3B). The identified protein contains a putative signal

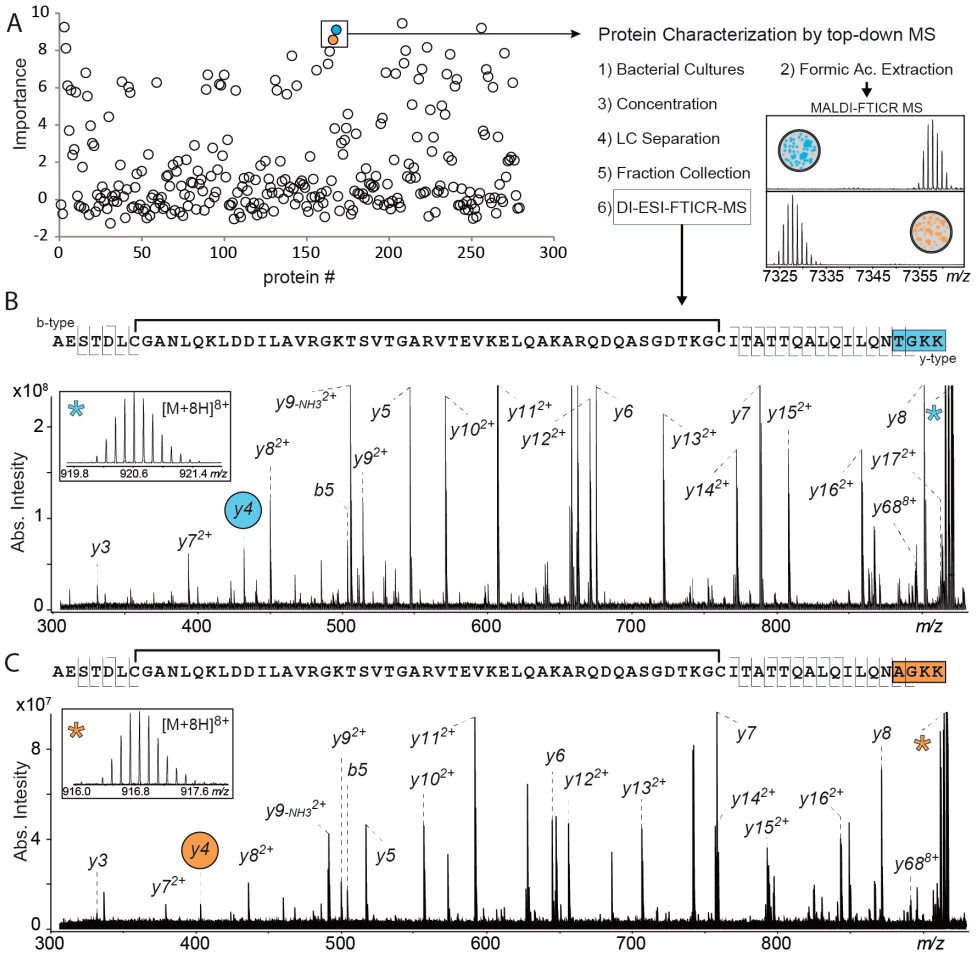

Figure 3: Top-down identification of two highly discriminatory proteins between different *P. aeruginosa* isolates.

A) plot illustrating the contribution of individual proteins on the separation of the *P. aeruginosa* clinical isolates into different clusters and the workflow for subsequent top-down protein identification. The inset shows the signal in MALDI-FTICR MS analysis of the two selected species between *m/z* 7320-7370. B and C: top-down ESI-CID-FTICR MS analysis of the two different proteins highlighted in A. The insets show the precursor ion signal in ESI-FTICR MS. Based on the CID spectra a possible sequence tag was generated. BLAST searches in the UniProt database led to the identification of two different isoforms of the same hypothetical protein with theoretical *m/z-values* of 7353.8493 $[M+H]^+$ and 7323.8388 $[M+H]^+$, respectively, including a disulphide bond in both.

peptide of 19 amino acids and the mature protein therefore starts at Ala-20 (Figure S2A in Supporting Information). Taking into account a disulfide bridge between the two cysteines, the theoretical *m/z*–value of this protein species is 7353.8493 $[M+H]^+$, which fits with the MALDI-FTICR and ESI-FTICR MS data. The full protein sequence allowed for the assignment of additional b- and y-ions (Figure 3B and Figure S3 in Supporting Information). The fact that no fragment ions were observed between the two cysteines hints to the presence of a disulfide bridge.

Interestingly, the initial sequence tag resulted in the identification of two isoforms of this protein only differing in position 86 (Figure S2B in Supporting Information). Either a Thr (as in the protein identified above) or an Ala is found in this position. Another discriminating protein (Figure 3A, orange dot and inset) at *m/z* 7323.9827 is present in the MALDI-FTICR MS data of different clinical isolates. This is 30.021 Da lower than the protein identified above and corresponds nicely to an Thr-Ala substitution (theoretical change 30.016 Da). This was confirmed with top-down MS/MS analysis of this protein species, selecting the ion at *m/z* 916.3668 $(M+8H)^{8+}$ in the corresponding chromatographic fraction from the extract of another clinical isolate. The fragmentation spectrum contained the same b-ions (*e.g.* b_5) but, the y-ions starting from y_4 were shifted with the expected mass (Figure 3C).

Subsequently, we checked all our MALDI-FTICR MS spectra and found that the presence of the two isoforms is mutually exclusive. In fact, the Thr-86 variant, shown in blue in Figure 3, was found exclusively in the protein profiles of strains of cluster 1, whereas the Ala-86 variant was found only in the strains of clusters 2 and 3. This further emphasizes that this protein contributes to the differentiation of the clusters. No clear protein homologues of this protein with an annotated function could be found in other species. Using the I-TASSER server for protein function prediction[39], some indications for a function in metal binding were retrieved. This warrants further investigation however, especially since under this scenario the cysteine residues would not be conserved. Of note, in one of the spectra (KML 08), none of the two isoforms was resolved. This strain was still clearly clustered within cluster 1, demonstrating that other features were also important for the classification.

Conclusions

The standardization of MALDI-TOF MS platforms has paved the way for routine applications of mass spectrometry in clinical microbiology laboratories. Due to intrinsic low resolution and moderate sensitivity, bacterial typing using MALDI-TOF MS is, as also shown in this paper, limited. In this study we used ultrahigh resolution MALDI-FTICR MS to analysis in more detail bacterial proteins from *P. aeruginosa* isolates. This is a novel application of ultrahigh resolution profiling, which maintains all the speed advantages of MALDI-TOF bacterial protein profiling, while being able to reliably discriminate between different strains of *P. aeruginosa*. In fact, using this new method we were able to cluster 18 *P. aeruginosa* clinical isolates in a similar manner as was obtained using AFLP analysis. The additional power of the FTICR platform was further demonstrated by the *de-novo* sequencing of two proteoforms of a hypothetical protein that contributes significantly to the discrimination of the different clusters. However, further investigation is needed to assess whether any of the analyzed bacterial proteins from cluster 1 can be linked to the antibiotic resistance.

In the future when expanding on this proof-of-principle experiment, we believe that this approach can be used for the rapid typing of clinical isolates, especially in the context of hospital outbreaks where fast and appropriate infection control management is essential.

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Supplemental data

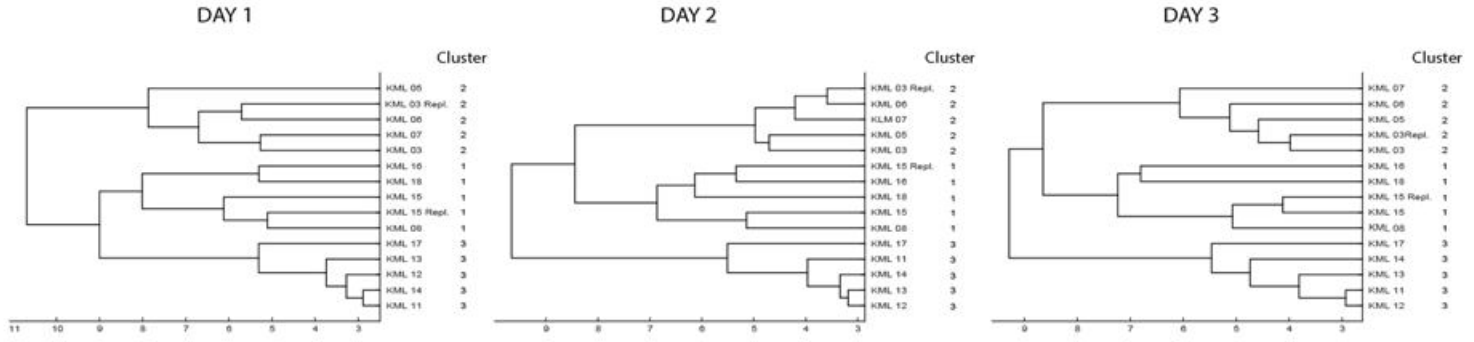


Figure S1. Clustering of *P. aeruginosa* clinical isolates following analysis on different days.

Day-to-day repeatability experiment of the clustering analysis based on MALDI-FTICR MS profiles. On three consecutive days extracts were prepared and measured. Clustering in three groups was consistent in these independent experiments.

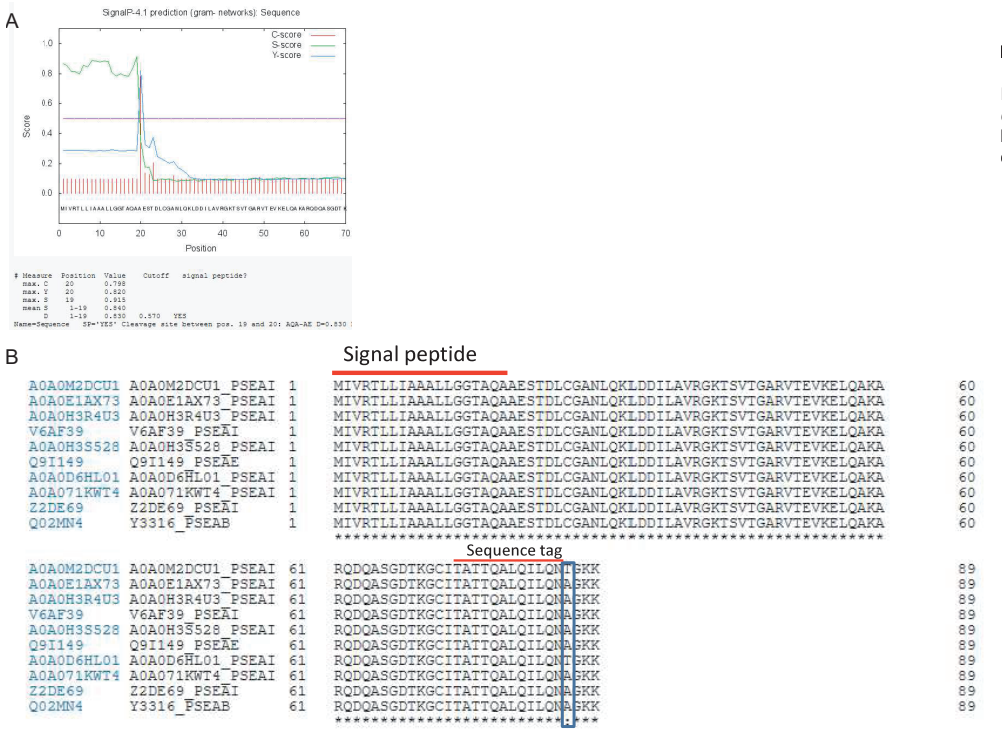


Figure S2. Signal peptide analysis and isoform comparison of the P. aeruginosa hypothetical protein identified by topdown ESI–CID–FTICR MS.

A: Predicted cleavage site of the signal peptide of the hypothetical protein identified by topdown ESI-CID-FTICR MS (Figure 3). B: Annotation of the signal peptide, the de novo obtained sequence variation at position 86 of the hypothetical protein where either a Thr or Ala is present. Sequences were obtained from the Uniprot database.

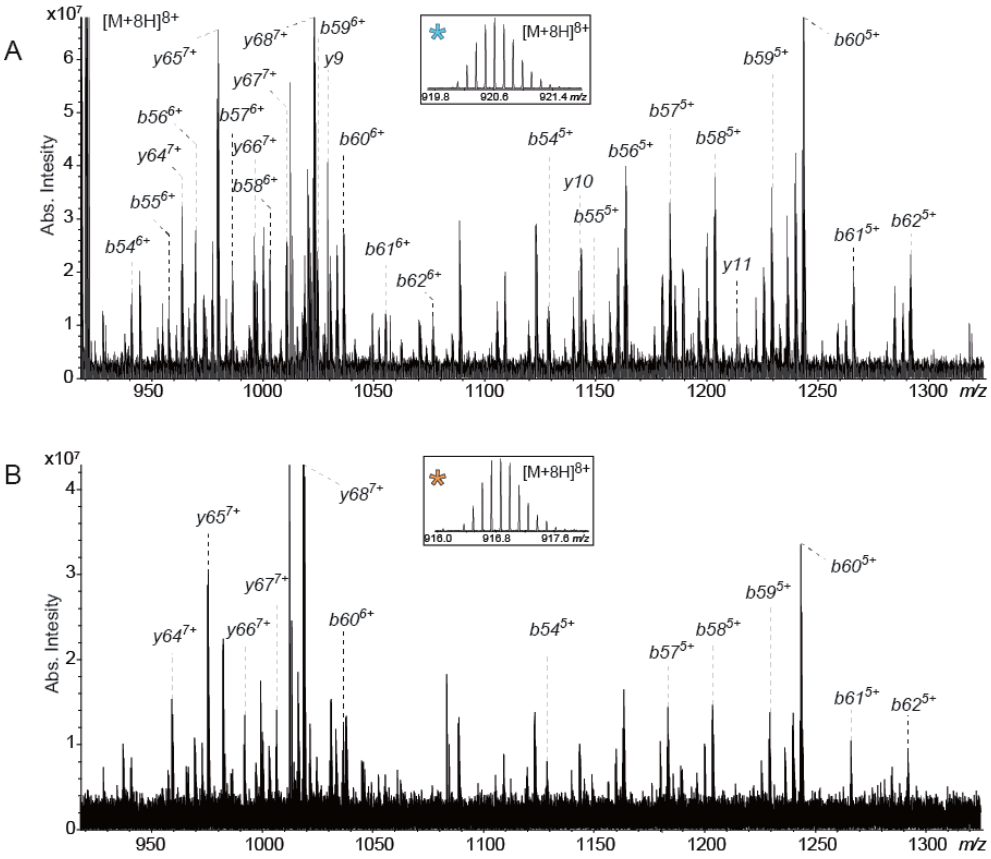
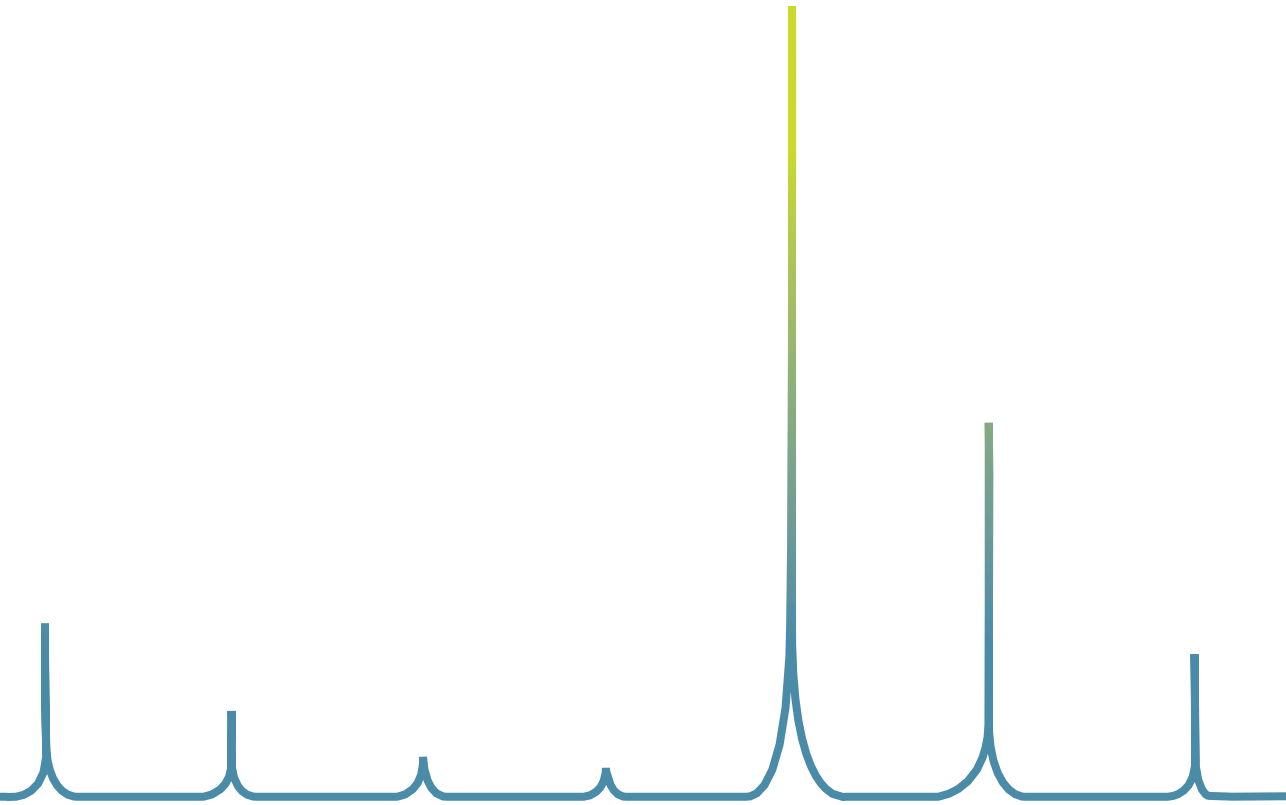


Figure S3. Top–down identification of two highly discriminatory proteins between different *P. aeruginosa* isolates.

Enlargement of ESI-CID-FTICR spectra of the discriminant proteins highlighted in blue (A) and in orange (B) in Figure 3A.



CHAPTER 5

Proteomic identification of Axc, a novel beta-lactamase with carbapenemase activity in a meropenem-resistant clinical isolate of *Achromobacter xylosoxidans*

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Abstract

The development of antibiotic resistance during treatment is a threat to patients and their environment. Insight in the mechanisms of resistance development is important for appropriate therapy and infection control. Here, we describe how through the application of mass spectrometry-based proteomics, a novel beta-lactamase Axc was identified as an indicator of acquired carbapenem resistance in a clinical isolate of *Achromobacter xylosoxidans*.

Comparative proteomic analysis of consecutively collected susceptible and resistant isolates from the same patient revealed that high Axc protein levels were only observed in the resistant isolate. Heterologous expression of Axc in *Escherichia coli* significantly increased the resistance towards carbapenems. Importantly, direct Axc mediated hydrolysis of imipenem was demonstrated using pH shift assays and ^{1}H-NMR, confirming Axc as a legitimate carbapenemase. Whole genome sequencing revealed that the susceptible and resistant isolates were remarkably similar.

Together these findings provide a molecular context for the fast development of meropenem resistance in *A. xylosoxidans* during treatment and demonstrate the use of mass spectrometric techniques in identifying novel resistance determinants.

Introduction

Development and spread of antibiotic resistance by pathogenic microorganisms is an increasing healthcare problem. Moreover, certain resistance determinants spread readily[1,2], while the introduction of novel antibiotics is lagging behind. Several clinically important classes of antimicrobials such as the beta-lactams, target the bacterial cell wall[3]. Resistance to beta-lactams can be mediated by beta-lactamases that are capable of hydrolysing the beta-lactam ring. Following the initial introduction of penicillin, second and third generation beta-lactams have been developed which, in turn, triggered the selection of beta-lactamases with broader specificities. Carbapenem treatment is often used as a last resort, since extended-spectrum beta-lactamases (cephalosporinases) are becoming more prevalent in Gram-negative bacteria. The emergence and spread of carbapenemases such as class A KPC[4], a number of metallo beta-lactamases[5,6] (class B: IMP, VIM, NDM) and class D oxacillinases such as OXA-48[7], in combination with other resistance mechanisms[8], can jeopardize carbapenem efficacy, leaving little or no treatment options for patients.

Achromobacter xylosoxidans is a rod shaped aerobic non-fermentative Gram-negative bacterium. It is widespread in the environment and generally considered as an opportunistic pathogen. Chronic infections with *A. xylosoxidans* are problematic in cystic fibrosis patients[9,10] but reported prevalence numbers vary greatly (3-30%)[11,12]. Moreover, bacteremia as a result of *A. xylosoxidans* can occur in immunocompromised patients[13]. *A. xylosoxidans* is notorious for its intrinsic high level of resistance, especially towards penicillins and cephalosporins[14-16]. In general, carbapenem resistance in *A. xylosoxidans* is not widespread and as a result meropenem treatment is routinely applied, even in the case of recurring infections[17,18]. Though carbapenem resistance is observed, specifically for meropenem[19], there are few reports on the mechanism of carbapenemase resistance in *A. xylosoxidans.* Notable exceptions are the plasmid-encoded carbapenemase IMP[20,21] and the chromosomally encoded class D beta-lactamase OXA-114[16], that show low level carbapenemase activity. A comparative genomic exploration of two *A. xylosoxidans* isolates revealed many genes that could be involved in drug resistance, such as efflux pumps and β-lactamases. However, most of these genes were conserved between carbapenem susceptible and resistant strains, highlighting the difficulty in translating genomic data to the observed resistant phenotypes[22].

In this study, two clinical isolates of *A. xylosoxidans* from an immunocompromised patient with pneumonia were investigated. The initially cultured isolate from the respiratory tract was susceptible to meropenem and treatment was started accordingly. However, a subsequent meropenem resistant isolate was obtained from a blood culture after treatment failure. Since PCR analysis was negative for known carbapenemases, we performed a proteomic analysis which revealed the novel beta-lactamase Axc as highly abundant in the meropenem-resistant, but not in the susceptible isolate. Axc expression led to an increase of minimal inhibitory

concentrations for carbapenems when introduced in a susceptible *Escherichia coli* strain and direct carbapenemase activity of Axc was demonstrated using *in vitro* imipenem conversion assays. Interestingly, the resistant and the susceptible clinical isolates are genetically almost identical, emphasizing the importance of mass spectrometry as a technique to investigate carbapenem resistance in *A. xylosoxidans*.

Results

Development of meropenem resistance in *Achromobacter xylosoxidans* during treatment

An antibiogram of a clinical isolate of *Achromobacter xylosoxidans* from a patient treated for pneumonia (see Methods for details about patient history and treatment) revealed a multi-resistant character, but it was susceptible to meropenem (Supplemental Table 1). Hence, meropenem treatment was initiated. During treatment, the patient developed a pneumothorax and died from septic shock 6 days later. An antibiogram of a blood culture from a sample taken one day before the patient's death revealed a meropenem-resistant *A. xylosoxidans* phenotype (Supplemental Table 1). Subsequently, pure cultures from the first and second clinical isolate were prepared (AchroS and AchroR, respectively). In line with the antibiogram analysis described above, Etests showed that both isolates were resistant to imipenem (MIC > 32 mg/L for both) but differed in their susceptibility towards meropenem (MIC of 0.0094 mg/L (first isolate, AchroS) and 2 mg/L (second isolate, AchroR), respectively). An in-house multiplex PCR assay (based on a published PCR[34]) failed to detect common carbapenemases (KPC, IMP, VIM, NDM and OXA-48, data not shown), suggesting that the change in resistance is not mediated by these enzymes. This finding prompted us to perform a comparative proteomic analysis of the two isolates to attempt to identify the meropenem resistance mechanism.

Comparative proteomic analysis shows differential levels of the beta–lactamase Axc

Protein extracts of the meropenem-susceptible and resistant *A. xylosoxidans* isolates (AchroS and AchroR) were first analysed by SDS-PAGE. Since no major visual differences were observed (Supplemental Figure S1A), all bands were excised and processed for a bottom-up LC-MS/MS proteomic analysis. In total, 2276 unique proteins were identified, of which 1519 proteins were common to both isolates, while 223 and 534 were only found in the susceptible and resistant isolates, respectively. For a semi-quantitative analysis, the spectra assigned to peptides belonging to a certain protein were counted and compared between the two different isolates (Supplemental Figure S1B and S2, Table S2). Of the uniquely observed proteins, most were proteins with low spectral counts (often single peptide identifications), likely resulting from sampling bias of low abundant proteins. One protein was observed with 102 spectra in the resistant isolate (AchroR) but none in the susceptible isolate (AchroS). This

protein, hereafter called Axc (for *Achromobacter xylosoxidans* carbapenemase, GenBank ID: MF767301), is a putative PenP class A beta-lactamase (COG2367/pfam13354).

To confirm that Axc was highly abundant in the resistant isolate in comparison to the susceptible isolate, a second proteomic analysis was performed on whole cell protein extracts that were digested in-solution and analysed without any prior fractionation. Spectral count analysis resulted in a cumulative quantification of 1342 different proteins, of which 981 were found in both isolates, but 196 and 165 were uniquely quantified in the susceptible and resistant isolates, respectively. The spectral count plot reflects the high similarity of the two clinical isolates, with the vast majority of the proteins distributed along the diagonal (Figure 1A, Table S2, Supplemental figure S2). In accordance with the data described above, Axc was the most prominent outlier in the resistant clinical isolate. A number of Axc tryptic peptides (Figure 1B) was clearly visible in the LC-MS/MS analysis of the resistant but not the susceptible isolates (Figure 1C). Of note, cells used for these analyses were grown in the absence of meropenem, so the high level of Axc in the resistant isolate was independent of antibiotic pressure.

Axc is present in meropenem resistant and susceptible *A. xylosoxidans* isolates

To investigate whether A. xylosoxidans acquired the axc gene in the course of the treatment, we performed a PCR for the axc open reading frame on both the resistant and susceptible isolates AchroS and AchroR. This analysis demonstrated that axc was present in both clinical isolates (Supplemental Figure S3A). Moreover, Sanger sequence analysis demonstrated that the sequence of axc was identical in both isolates (data not shown), indicating that the observed meropenem resistance was not likely caused by an alteration of protein function.

A database search revealed that the axc gene is not present in all A. xylosoxidans strains (Figure 2A). Like in our clinical isolates, axc is present in the NH44784-1996 strain (ENA assembly GCA_000967095.1) 35 but not in the strains NBRC 15126/ATCC 27061 (ENA assembly GCA_000508285.1) and C54 (ENA assembly GCA_000758265.1) for instance. In those strains that contain axc, the gene is located next to a putative LysR-type transcriptional regulator (Pfam 03466), hereafter axcR (for axc-associated regulator). Additional PCR and Sanger sequencing experiments verified that the intergenic regions between axc and axcR in AchroS and AchroR were identical, However, they differed at two positions with the intergenic region between axc and axcR in strain NH44784_1996 (Supplemental Figure S3B).

The results showed a high degree of similarity between the meropenem resistant and susceptible strain, raising the possibility that these two strains represented a clonal complex. To further explore the relatedness of both isolates, whole genome sequencing (WGS) analysis was performed. This showed that both patient isolates were highly similar, with only one single-nucleotide polymorphism (SNP) within the gene encoding AxyZ 36. This SNP was

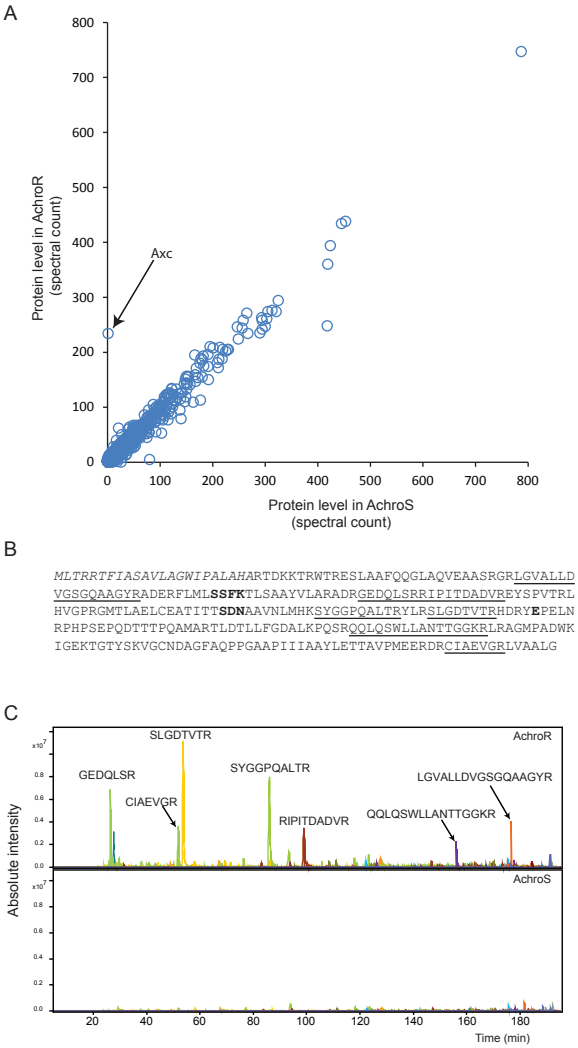

Figure 1: Comparative proteomic analysis of meropenem resistant and susceptible *Achromobacter xylosoxidans* clinical isolates

A: Tryptic digests of protein extracts of the meropenem resistant (AchroR, Table 1) and susceptible (AchroS, Table 1) isolate were analysed by LC-MS/MS. Spectra were assigned to peptides based on database searching. Identified spectra were then assigned to the corresponding proteins and the total number of spectra per protein were counted. Each circle represents one protein with the number of spectra observed in the resistant and the susceptible isolate. Hence, proteins on the diagonal were observed in similar counts in both isolates. Axc (arrow), a classA PenP-family beta-lactamase, is the most prominent outlier. See also Table S2 and Supplemental figure S2.

B: The full amino acid sequence of Axc, with the peptides identified by LC-MS/MS analysis underlined. Conserved residues from serine beta-lactamases, Ser-X-X-Lys, Ser-Asp-Asn and the active site Glu, are in bold (37).

C: Extracted ion chromatograms of *m/z* values corresponding to tryptic peptides of Axc in the meropenem resistant isolate (AchroR, upper panel) and susceptible isolate (AchroS, lower panel). The corresponding tryptic peptides are indicated above the corresponding peaks.

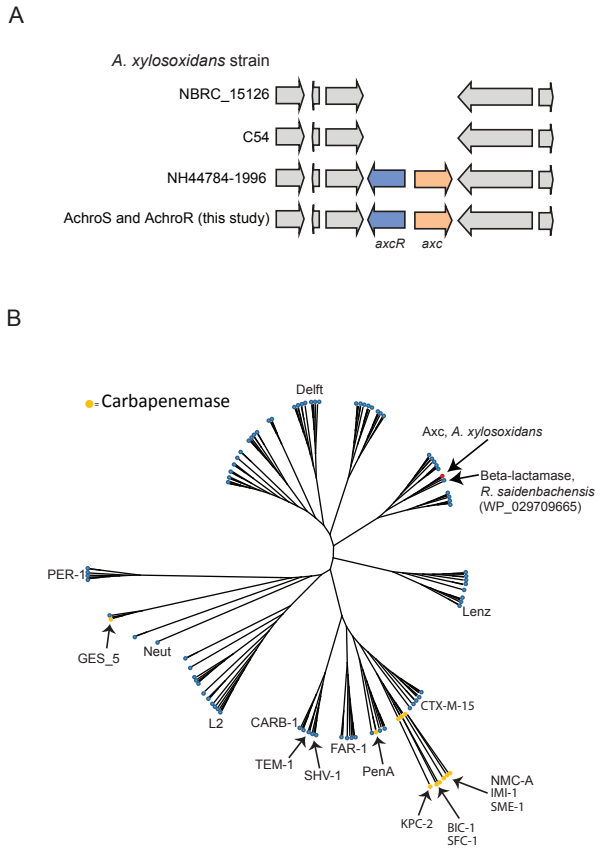

Figure 2: Genomic context of *axc* in *Achromobacter xylosoxidans* strains and comparison of Axc with other class A beta–lactamases

A: *Axc* (a putative PenP class A beta-lactamase, 1.17 e^{-54}) and the gene encoding its putative transcriptional repressor (*axcR*), were found in both clinical isolates (AchroS and AchroR, Table 1). Three other fully sequenced genomes of *Achromobacter xylosoxidans* were examined for the presence of *axc*; NH44784_1996 (ENA assembly GCA_000967095.1), C54 (ENA assembly GCA_000758265.1) and NBRC_15126/ATCC27061 (ENA assembly GCA_000508285.1). Only within the strain NH44784_1996 (used as a reference to search our proteomics data), *axc* and the putative regulator are also present.

B: Unrooted cladogram obtained for 176 class A beta-lactamases including Axc**.** The class A β-lactamase protein sequences of Gram negative bacteria were obtained by querying the refseq_protein database using Blastp (<1e-10, http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) and a consensus β-lactamase-alignment (37). Duplicate sequences and sequences causing a strong overrepresentation of branches produced in the tree were removed. Names of known carbapenames (orange) and names of identifier of branches (37) are indicated. Axc is indicated with a red dot. The closest homologue to Axc, a β -lactamase from *R. saidenbachensis* (WP_029709665), is also indicated.

confirmed by PCR and subsequent Sanger sequencing, ruling out the possibility that this was an artefact of the assembly procedure. Moreover, axc and its putative regulator axcR were found to be located in the same genomic region as in NH44784_1996 strain (Figure 2A). Overall, the genome sequences suggest that both isolates are clonal, and that the meropenem resistance evolved within the same strain during the course of treatment.

To demonstrate how Axc is related to other class A beta-lactamases, we compared the Axc amino acid sequence with the sequence of another 176 representatives of this family using an alignment consensus based on a report by Walther-Rasmussen and Hoiby 37. The resulting unrooted cladogram shows that Axc is most closely related to a class A beta-lactamase of Rhodoferax saidenbachensis (WP_029709665, Figure 2B). Only a limited number of class A beta-lactamases have activity towards carbapenems, but none of these cluster with the Axc sequence (Figure 2B).

Functional characterisation of Axc

To establish whether Axc indeed has activity towards carbapenems, Axc was expressed in a heterologous host and hydrolysis of carbapenems was measured indirectly and directly.
The *E. coli* C43 strain, suitable for the expression of toxic proteins[30], is susceptible to carbapenems. We generated a derivative of C43 that allows for IPTG-dependent expression of a plasmid-based copy of *axc* (*E. coli*_Axc (JC107)). Susceptibility testing for imipenem and meropenem showed that the MICs for these carbapenems increased 8-fold, following induction of *Axc* expression (Table 2). Though these levels were lower than for the positive control (KPC) for our assay, they were specific for Axc as the expression of an unrelated protein (PPEP-1[31]) did not lead to an increase in MIC values (Table 2). Thus, expression of Axc is correlated to resistance towards carbapenems.

To directly demonstrate carbapenemase activity, hydrolysis of imipenem was monitored *in vitro* through colorimetric assays (Figure 3A) and ^{1}H-NMR (Figure 3B). Imipenem hydrolysis results in the formation of a carboxylic acid, and monitoring the accompanying pH drop colorimetrically is a well-established method for the detection of carbapenemase activity[32,38]. Indeed, hydrolysis of imipenem was readily observed using KPC cells (without IPTG). Consistent with our previous results, imipenem hydrolysis was observed for *E. coli* cells harbouring the Axc expression plasmid (*E. coli*_Axc) grown in the presence, but not in the absence, of IPTG. As before, these results were specific for Axc, as induction of PPEP-1 expression (*E.coli*_control) did not result in imipenem hydrolysis (Figure 3A). In a parallel assay, ^{1}H-NMR was used to directly observe the opening (hydrolysis) of the lactam ring in imipenem (Figure 3B). The chemical shifts of the peaks change as a result of this hydrolysis, with the protons closest to the ring opening undergoing the largest change. The H-6 proton of imipenem generates an adequately resolved multiplet at 3.42 ppm that decreases in intensity upon hydrolysis. Concomitantly, the doublet generated by the protons of the

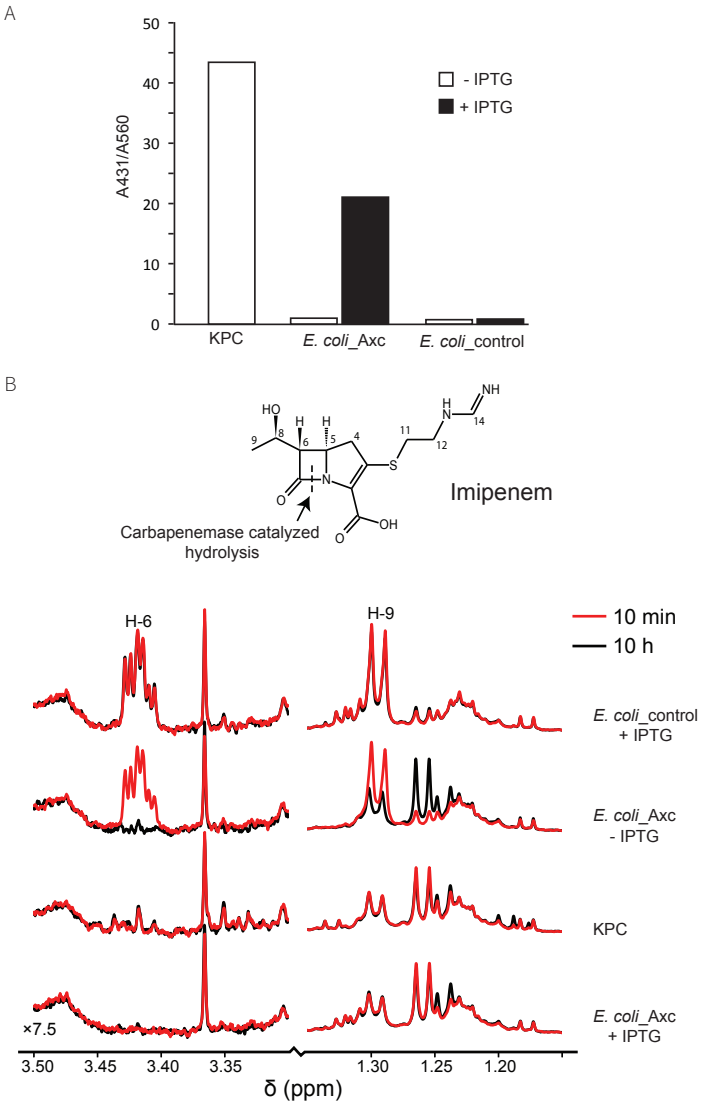

Figure 3: Axc has carbapenemase activity.

A: Axc was expressed in *E. coli* and cell extracts were tested for the ability to hydrolyse imipenem. Bars show the A431/A560 ratio, which is a measure for the shift in pH due to imipenem hydrolysis.
B: NMR-based identification of imipenem hydrolysis by Axc. The structure of imipenem is shown, with the proton numbering used in the spectrum. The line color indicates the incubation time (red, 10 min at room temperature or black, 10 hrs at 6 °C). Imipenem hydrolysis is accompanied by the loss of the H-6 multiplet at 3.4 ppm, and a shift in the H-9 doublet, resulting in a decrease of the doublet at 1.3 ppm.

Strains used (see also Table 1): KPC (JC113): Carbapenem resistant *Klebsiella pneumoniae*. *E.coli* Axc (JC107): *E. coli* strain C43(DE3), containing plasmid pET21-Axc; *E.coli*_control (JC108): *E. coli* strain C43, containing plasmid pET21-PPEP-1 (Pro-Pro endopeptidase 1). IPTG: isopropyl β-D-1-thiogalactopyranoside

ºmethyl group (H-9) move upfield, resulting in a decrease of the doublet at 1.3 ppm. After 10 min incubation of bacterial cells with imipenem, hydrolysis of imipenem was observed with *E. coli*_Axc grown in the presence, but not in the absence, of IPTG. Hydrolysis was also apparent for KPC, but not for *E. coli* cells expressing PPEP-1 (*E.coli*_control)), even after long incubation times (10h). Under these conditions, imipenem hydrolysis was also observed for samples with *E. coli* cells harbouring the Axc expression plasmid grown in absence of IPTG, due to leaky expression from the inducible promoter.

Taken together, our data establish that Axc has carbapenemase activity.

Discussion

In this paper we identified a new resistance mechanism that explains the difference in meropenem susceptibility of two clinical isolates of *A. xylosoxidans* that were collected during treatment. Using a combination of comparative proteomic analyses and functional assays, we have shown that the class A beta-lactamase Axc is highly abundant in the meropenem resistant isolate in comparison to the susceptible isolate and that Axc has carbapenemase activity.

Detection of carbapenemases from sequence data is challenging. First, carbapenemases belong to different subgroups of beta-lactamases which have probably evolved by convergent evolution. Although sequence identities are moderate, most of the class A carbapenemases have a disulphide bridge between Cys-69 and Cys-238, but this is dispensable for activity against carbapenems[39] and our data show that Axc does not contain these residues. Next, some studies also revealed a mechanism where beta-lactam trapping, without actual degradation, can be involved in resistance towards carbapenems when levels are sufficiently high. In such cases, concomitant loss of porins is often observed[40,41]. Thus, it is crucial to determine whether a beta-lactamase actually induces hydrolysis of carbapenems. Our NMR and pH-shift analyses data clearly demonstrate Axc-mediated opening of the beta-lactam ring in imipenem. Finally, many unexplained mechanisms of carbapenem resistance remain. For instance, a recent study demonstrated plasmid derived carbapenem resistance in *Klebsiella pneumoniae* strains which could not be explained by the most commonly found carbapenemases (KPC-type). Even though none of the plasmid-encoded genes were obvious candidates for the observed resistance towards carbapenems, several TEM-homologs were detected[42]. Though the prediction of the activity of a certain beta-lactamase against carbapenems is not straightforward studies such as the present one highlight that mass spectrometry approaches can be used to gain insight in the mechanism of action and role of specific proteins in the observed phenotypes.

We do not know whether the high level of Axc is the only mechanism conferring meropenem resistance to our isolate of *A. xylosoxidans*. When expressed from an inducible promoter, Axc confers moderate resistance to carbapenems to *E. coli*; MIC values compared to the KPC strain suggest that Axc has a lower efficiency than KPC, but may also indicate lower overall levels of expression. Differing efficiencies in carbapenemases are well documented, to the point where the activity of a specific class, such as OXA-48, is difficult to detect but of great clinical importance[43]. Full biochemical characterization of Axc, including kinetic experiments, is subject to further study. Such experiments, in combination with crystallography analysis, will provide more insight in the activity of Axc against different beta-lactams and could resolve the structural characteristics of the binding pocket which facilitates its activity towards carbapenems. We note, however, that two other changes in the antibiogram between the meropenem susceptible and resistant isolates involve beta-lactam antibiotics. Augmentin (amoxicillin/clavulanate) resistance changed from intermediate (8 mg/L) to resistant (>32 mg/L), and piperacilline/tazobactam from susceptible (<=4 mg/L) to intermediate resistance (8 mg/L). This suggests that Axc may have a broad substrate specificity and is insensitive to inhibition by clavulanate and tazobactam. Strikingly, both the meropenem-susceptible and meropenem-resistant isolates were resistant to imipenem (MIC values higher than the maximum concentration tested (32 mg/L)). This indicates that, notwithstanding the activity of Axc towards imipenem as presented here, imipenem resistance in the clinical isolates is not dependent on Axc. Differences in the sensitivities towards different carbapenems results from the chemical differences between the individual drugs[44] and are often linked to the differential permeability of the outer cell membrane[45,46].

The regulatory mechanism leading to higher levels of Axc expression are unclear. Sequence analyses showed that the meropenem susceptible and resistant *A. xylosoxidans* clinical isolates are highly similar, with no differences in the *axc* promotor and coding sequence, nor in its putative regulator AxcR and the *axc-axcR* intergenic region. The only SNP we identified is located in the gene encoding AxyZ, the TetR-type repressor of the *axyXY-oprZ* operon[47]. This leads to an amino acid substitution (V29G) in a region of AxyZ that is involved in DNA binding in other members of TetR family[48]. AxyX, AxyY and OprZ form an efflux pump of the resistance-nodulation division (RND) family and are predominantly found in aminoglycoside resistant *Achromobacter* species[49-51]. A recent paper showed higher expression levels of *axyY* in *A. xylosoxidans* strains containing AxyZ_Gly29, suggesting that this mutation leads to reduced repression of AxyZ[52]. Closer inspection of our proteomics data indicates that also AxyX and AxyY are more abundant in the resistant isolate (spectral counts of 62 *vs* 21 for AxyX and 10 *vs* 2 for AxyY in the results presented in Figure 1 (see Table S2)). However, the difference is not as pronounced as found for Axc and more accurate quantitative proteomics experiments have to be performed to validate these data.

Mutations in TetR-like repressors have been linked to differences in carbapenem resistance[53]. For example, a 162 bp deletion in *axyZ* has been identified in certain carbapenem resistant strains[54]. However, even though AxyZ_Gly29 leads to higher expression of *axyY*, resulting in higher MIC values for aminoglycosides, fluoroquinolones and tetracyclines, no correlation between *axyY* expression and meropenem resistance was observed[52]. In line with this observation, a deletion of *axyY* in several *A. xylosoxidans* strains is reported to result in only a modest increase in the susceptibility towards carbapenems[47]. Taken together, it is likely that AxyXY-OprZ per se contributes little if any to the meropenem resistance phenotype of our clinical isolate. Instead, our data show a critical role for Axc and suggest that *axc* expression is regulated by AxyZ. If this is indeed the case, we postulate that the increase in the meropenem MIC for the ACH-CF-911$_{V29G}$ strain[52] is accompanied by increased Axc levels.

From the clinical perspective, the development of resistance to meropenem within days following meropenem treatment is remarkable. Previous longitudinal analyses of different *A. xylosoxidans* isolates from one patient have revealed large phenotypic and genetic differences, for example in the resistance towards different classes of antibiotics but they were generally performed over longer time periods[35,53,54]. Such changes are believed to be the result of adaptive evolution of the initial strain which infected the patient, but there is also evidence that genetically different strains of *A. xylosoxidans* can co-exist within the same chronically infected individual[55]. Though we cannot exclude a co-infection, it is likely that the mutation in *axyZ* in our case occurred during treatment.

Finally, from a diagnostic point of view, the presence of *Axc* in both the sensitive and resistant *A. xylosoxidans* isolates complicates straightforward detection by molecular methods in the future and warrants detection based on protein abundance levels. Even though more elaborate mass spectrometry-based platforms clearly have potential for the detection of resistance and virulence proteins[23,56], as also exemplified in our study, it will take more time and effort before they could find their way into the medical microbiology laboratory to complement the now well-established application of MALDI-ToF-MS for bacterial species identification.

Methods

Patient history

All procedures performed in studies involving human participants were in accordance with the ethical standards of LUMC Medical Ethical Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

A 65-year old patient, diagnosed with chronic lymphocytic leukemia in 1989, underwent a non-myeloablative stem cell transplantation in July 2014. The patient was discharged on July 25th but readmitted on August 1st with a mucositis-associated neutropenic fever and blood cultures positive for *Gemella haemolysans*. The patient was treated with cefuroxim intravenously but developed pneumonia one week later. Analysis of a sputum sample showed a microscopically and culture confirmed fungal infection and abundant growth of a Gram-negative bacterium. This bacterium was identified as *A. xylosoxidans* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The patient was treated with liposomal amphotericin B for the fungal infection, but this did not result in improvement of the patient's status. Due to clinical deterioration the patient received ceftazidim (3 dd 1 gram intravenously) between September 3rd and 9th to treat the ceftazidim sensitive *A. xylosoxidans* isolate. On September 10th, the antibiogram (Supplemental Table 1), revealed a multi-resistant character for the *A. xylosoxidans* isolate, but it was susceptible to meropenem. Hence, meropenem treatment was initiated (4 dd 1 gram intravenously) on September 10th. During treatment the patient developed a pneumothorax and died from septic shock 6 days later. Because of this, informed consent of the patient was not possible to obtain. We therefore anonymized all patient data and presented the data in such a form that it is not traceable to the patient.

Materials

MilliQ water was obtained from a Q-Gard 2 system (Merck Millipore, Amsterdam the Netherlands). Acetonitrile of LC-MS grade was obtained from Biosolve (Valkenswaard, the Netherlands). Porcine trypsin was purchased from Promega (Madison, WI). If not indicated otherwise, chemicals were from Sigma Aldrich (St Louis, MN, USA).

Susceptibility profiling of clinical isolates

The minimum inhibitory concentrations (MICs) for different antibiotic compounds on the clinical isolates (Clinical IDs: M 14073954-7 (first isolate), M 14076260-2 (second isolate)) were initially determined using a Vitek-2 system (bioMérieux, Marcy-l'Étoile, France). Several colonies of plate grown cultures were inoculated and suspended in 0.45% sterile physiological saline solution. Suspensions for testing had densities between of approx. 0.5 McFarland standards. The testing procedure was performed according to the manufacturer's instructions.

Pure cultures of the susceptible and resistant isolates (AchroS and AchroR, respectively (Table 2)) were subjected to further susceptibility testing and used for the proteomic and genomic analyses. Etests (bioMérieux) were performed according to the recommendations from EUCAST (http://www.eucast.org/clinical_breakpoints/). EUCAST does not provide recommendations for the interpretation of MIC values or clinical breakpoints for *Achromobacter xylosoxidans*. Therefore, scoring was performed using locally developed

protocols that are based on clinical breakpoints for other non-fermentative Gram-negative rods.

Mass spectrometry–based proteomics

Cells were grown in BHI (Oxoid, Basingstoke, UK). Cells were collected by centrifugation (4000 x *g*, 5 minutes) from 1 mL cell culture and washed with phosphate- buffered saline (PBS, pH 7.4). Pellets were stored at -80 °C until further use.

Two different proteomics experiments were performed. For the first, cell extracts from *A. xylosoxidans* strains (AchroS and AchroR, Table 2) were prepared in LDS (Lithium dodecyl sulphate) sample buffer (Novex, Thermo Scientific) and put at 95 °C for 5 minutes for cell lysis and protein extraction. Proteins were separated on Novex precast 4–12% Bis-Tris gels (Thermo Scientific, Waltham, MA, USA) with MOPS (3-(N-morpholino)propanesulfonic acid) running buffer (Thermo Scientific). After overnight staining using a Colloidal Blue Staining Kit (Thermo Scientific), destained gel lanes were processed into 31 slices per lane. Gel pieces were sequentially washed with 25 mM ammonium bicarbonate and acetonitrile. Reduction and alkylation were performed with dithiothreitol (DTT, 10 mM, 30 minutes at 56 °C) and iodoacetamide (IAA, 55 mM, 20 minutes at room temperature) respectively. Following several washes with 25 mM ammonium bicarbonate and acetonitrile, bands were overnight digested with trypsin (12.5 ng/mL in 25 mM NH_4HCO_3). Digest solutions were lyophilized and reconstituted in 0.5% trifluoroacetic acid (TFA). Nano-LC separation was carried out using a Ultimate 3000 RSLCnano System equipped with an Acclaim PepMap RSLC column (C18, 75 µm x 15 cm with 2 µm particles, Thermo Scientific) preceded by a 2 cm Acclaim PepMap100 guard column (Thermo Scientific). Peptide elution was performed by applying a mixture of solvents A and B with solvent A being 0.1% formic acid (FA) in water and solvent B 0.1% FA in 80 % acetonitrile (ACN). Peptides were eluted from the column with a multi-step gradient from 5% to 55% solvent B in 55 minutes (0-5 minutes 5%B, then in 15 minutes to 20%B and finally in 25 minutes till 55%B), at a constant flow rate of 300 nl min^{-1}. MS analysis was performed employing a maXis Impact UHR-TOF-MS (Bruker Daltonics, Bremen, Germany) in data dependent MS/MS mode in the *m/z* 150-2200 range. The source settings were as follows: voltage 1500 V, dry gas 3.0 $l.min^{-1}$, temperature 150 °C. Ten ions were selected at a time, based on relative abundance and subjected to collision-induced-dissociation with helium as collision gas. A 1 minute dynamic exclusion window was applied for precursor selection. The precursor isolation window and collision energy were *m/z* dependent.

For the second proteomics approach, in-solution digests were prepared as described previously[23]. In short, following cell disruption proteins were solubilized in 50% trifluoroethanol (TFE). Subsequent reduction with DTT and alkylation with IAA were performed prior to overnight tryptic digestion. Samples were lyophilized and reconstituted in 0.5% TFA for injection. The nano-LC system and solvents were the same as in the above experiment, but

using an Acclaim PepMap RSLC column (C18, 75 µm x 50cm with 2 µm particles, Thermo Scientific) with a 2 cm Acclaim PepMap100 guard column (Thermo Scientific). Peptides were eluted from the column with a multi-step gradient from 5% to 55% of solvent B in 180 minutes (0-5 minutes 5%B, then in 65 minutes till 13%B, then in 90 minutes till 35%B and finally in 20 minutes till 55%B), at a constant flow rate of 300 nL/min^{-1}. MS analysis was carried out as described above.

Mass spectrometry data export and spectral count analysis

Conversion of Bruker Impact files into mzXML format using CompassXport version 3.0.9, led to an initial total of 1,207,329 MS/MS and 444,565 MS/MS spectra, for the gel and total lysate based comparisons, respectively (in-solution total lysate digests were analysed twice and the data were merged). These spectra were searched using a concatenated forward and decoy strategy. The forward database was constructed from the 6386 unreviewed sequences from Uniprot for the organism *A. xylosoxidans* NH44784-1996 (November 2015) together with the cRAP contaminant sequences, as downloaded in January 2015. An in-house developed program, Decoy version V1.0.1-2-gfddc, that preserves homology, amino-acid frequency and peptide length distribution, was used with default flags to construct the decoy search space, which was concatenated to the forward sequences. The search against the resulting database was performed using Comet version 2014.02 rev.2, with precursor mass tolerance equal to 50 ppm and a fragment bin width of 0.05 Da, considering only fully tryptic digests with at most 2 missed cleavages. All cysteines were assumed carbamidomethylated, while methionine oxidation and N-terminal acetylation were regarded as variable modifications. The confidence of these results was assessed by means of Xinteract from the Trans Proteomics Pipeline suite version 4.8.0, retaining peptides longer than 6 amino acids and running in semi-parametric mode. A second in-house developed program, Pepxmltool version 2.5.1, was used to construct a protein quantification table based on spectral counting of only non-degenerate peptides (peptides mapping to a single protein) with corresponding q-value of at most 1%. Plots of the resistant versus susceptible quantifications of all proteins revealed for both methods a predominantly linear relationship, suggesting the applicability of an in-house developed program, Qntdiff version 0.1.1, to assign p-values to deviations from linear behaviour and provide lists of the most significantly differential protein expression levels between the two isolates. The programs Pepxmltool and Qntdiff are available from the authors on request.

Bacterial culture and genomic DNA preparation

AchroS and AchroR (Table 2) were cultured on trypcase soy agar plates (BioMérieux, Marcy l'Etoile, France), inoculated into liquid medium brain-heart-infusion (BHI) broth (Oxoid, Basingstoke, UK) and grown overnight (~16-hours) at 37 °C. Cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.4), and genomic DNA extraction was performed using a phenol-chloroform extraction as previously described[24].

Whole genome sequencing and SNP calling

Paired-end multiplex libraries were created as previously described[25]. Sequencing was performed on an Illumina Hiseq 2000 platform (Illumina, San Diego, Ca, USA), with a read-length of 100 basepairs. High-throughput *de novo* assembly of sequenced genome was performed as previously described[26,27]. The assemblies are then automatically annotated using PROKKA[28] with genus-specific databases from RefSeq[29]. To identify single nucleotide polymorphisms (SNPs), the Illumina sequence data of the meropenem-susceptible *A. xylosoxidans* isolate (AchroS) was mapped on the assembled genome of the meropenem-resistant isolate (AchroR) using SMALT software (http://smalt.sourceforge.net/), after which SNPs were determined as previously described[25].

PCR and heterologous expression of Axc

To corroborate the proteomics results and confirm results from the whole genome sequence analysis, the *axc* ORF, the *axc-axcR* intergenic region and part of the *axyZ* ORF were Sanger sequenced at a commercial provider (Macrogen, Amsterdam, the Netherlands). PCR products (for primers see Table 3) were sequenced using the same primers as those used for generating the product from genomic DNA isolated from the *A. xylosoxidans* isolates. We have submitted the Axc sequence to Genbank, ID MF767301.

To construct an *E. coli* strain expressing *Axc* with a C-terminal 6xHis-tag from an IPTG (isopropyl β-D-1-thiogalactopyranoside) inducible promoter, the *axc* open reading frame was amplified using primers Axfor2 and Axrev3 (Table 3), using Accuzyme polymerase (GC Biotech, Alphen aan den Rijn, The Netherlands) and genomic DNA from *A. xylosoxidans* (AchroR) as a template. The amplified PCR product was digested using NdeI (Bioké, Leiden, The Netherlands) and XhoI (Roche, Almere, The Netherlands), and cloned into similarly digested pET-21b(+), yielding plasmid pET21B/Axc. The *axc* expression region was confirmed using Sanger sequencing. Expression of Axc-his6 was carried out in *E. coli* C43(DE3)[30] in Luria-Bertani broth (Affymetrix, Cleveland, OH, USA) with ampicillin (50 µg/mL) and 1mM IPTG (GC Biotech, Alphen aan de Rijn, the Netherlands) for 3 hours at 37 °C and verified by immunoblotting using anti-His antibody (Agilent Technologies, Santa Clara, CA, USA).

Susceptibility testing of E. coli expressing Axc

Minimal inhibitory concentration (MIC) values for the carbapenems imipenem (Etest, BioMérieux) and meropenem (microbroth dilution) were established for *E. coli* C43/pET21B-Axc (strain JC107, Table 2) in the presence and absence of 1mM IPTG. For imipenem, cells were grown overnight in LB broth at 37 °C in the presence of ampicillin (50 µg/mL). The overnight cultures were diluted 1:100 in LB broth with ampicillin and grown to mid logarithmic phase (OD_{600nm}~ 0.5). Two hundred µl of bacterial culture was spread on LB-ampicillin (50 µg/mL) plates (with or without 1 mM IPTG) and an Imipenem Etest (BioMérieux) was applied. MIC values were determined after 24 hours incubation at 37°C. The meropenem MIC values were

established by microbroth dilution. Bacterial cultures in logarithmic phase (OD_{600nm} ~ 0.5) were diluted into LB-ampicillin medium to an OD_{600nm} of 0.05 in the presence or absence of 1mM IPTG and subsequently seeded in a 96-well plate. A two-fold serial dilution of meropenem (starting at 12.5 µg/mL) was made by adding equal amounts of meropenem (25 µg/mL) to the first row, from which a two-fold dilution series was made in the rest of the plate. Samples were investigated for growth by measuring the OD_{600nm} after 24 hours incubation at 37 °C, while shaking. The MIC was the lowest concentration of meropenem at which no growth was observed. As controls for our assays, a carbapenem resistant *Klebsiella pneumoniae* clinical isolate (KPC; JC113) and an unrelated expression construct (JC108; which expresses PPEP-1[31] in an IPTG-dependent manner in the same *E. coli* C43 background, Table 2) were included.

Colorimetric imipenem hydrolysis assay

Overnight bacterial cultures were diluted 100 fold in LB-ampicillin (50 µg/mL) medium and grown to exponential growth phase at 37 °C while shaking. At the time of induction, the OD_{600nm} was determined, the cultures were split in two and 1mM IPTG was added to one of the cultures, followed by a further incubation for 3 hrs at 37 °C while shaking. At T=3h, the OD_{600nm} was determined and cells were harvested by centrifugation (4000 g, 5 min) and stored at -20 °C overnight. Cells were resuspended in water to yield equal densities based on measured OD_{600nm} values. Then, 7.5 µL of bacterial suspension was mixed with 25 µL of imipenem/phenol red/$ZnSO_4$ solution (3 mg/mL imipenem, 0.35% (wt/vol) phenol red, pH 7.8, 70 µM $ZnSO_4$) and incubated at 37 °C for 1 hr. Conversion of imipenem leads to a pH drop that can be visualized by the color change of the buffer from red to yellow[32]. To quantify this effect, the UV-Vis spectrum was determined with a Nanodrop ND1000 spectrophotometer (Thermo Scientific) and the ratio between the absorption peaks at 431 and 560 nm was taken as a measure of imipenem hydrolysis.

NMR spectroscopy of imipenem conversion

All proton nuclear magnetic resonance (^{1}H-NMR) experiments were performed on a 600 MHz Bruker Avance II spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a 5-mm triple resonance inverse (TCI) cryogenic probe head with a Z-gradient system and automatic tuning and matching. All experiments were recorded at 310 K. Temperature calibration was done before each batch of measurements[33]. The duration of the π/2 pulses was automatically calibrated for each individual sample using a homonuclear-gated nutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head. The samples were prepared by adding 70 µL imipenem aqueous solution (5 mg/mL) to 280 µL milliQ water. This solution was mixed with 350 µL 75 mM phosphate buffer (pH 7.4) in water/deuterium oxide (80/20) containing 4.6 mM sodium 3-[trimethylsilyl] d4-propionate. Twenty µL of bacterial cell suspension were added and the sample was mixed. Samples were manually transferred into 5-mm SampleJet NMR tubes. The cell suspension samples were kept at 6 °C on a SampleJet sample changer while queued for acquisition. For water

suppression, presaturation of the water resonance with an effective field of γB1 = 25 Hz was applied during the relaxation delay. A 1D-version of the NOESY (Nuclear Overhauser effect spectroscopy) experiment was performed with a relaxation delay of 4 seconds. A NOESY mixing time of 10 ms was used during which the water resonance was irradiated with the presaturation RF field. After applying 4 dummy scans, a total of 98,304 data points covering a spectral width of 18,029 Hz were collected using 16 scans. The Free Induction Decay was zero-filled to 131,072 complex data points, and an exponential window function was applied with a line broadening factor of 0.3 Hz before Fourier transformation. The spectra were automatically phased and baseline corrected.

Bioinformatic analysis

Comparison of Axc with other class A beta lactamases was performed by multiple alignment using the Geneious 9.0 (Biomatters Ltd, Auckland, New Zealand)) software algorithm for Global alignment with free end gaps, cost Matrix Blosum62. The tree was then built using Jukes-Cantor genetic distance model with the Neighbor Joining tree build method.

Data availability

Illumina raw reads were deposited at the European Nucleotide Archive (ENA). Study ID: PRJEB19781. Sample IDs: ERS1575148 (AchroR) and ERS1575149 (AchroS).

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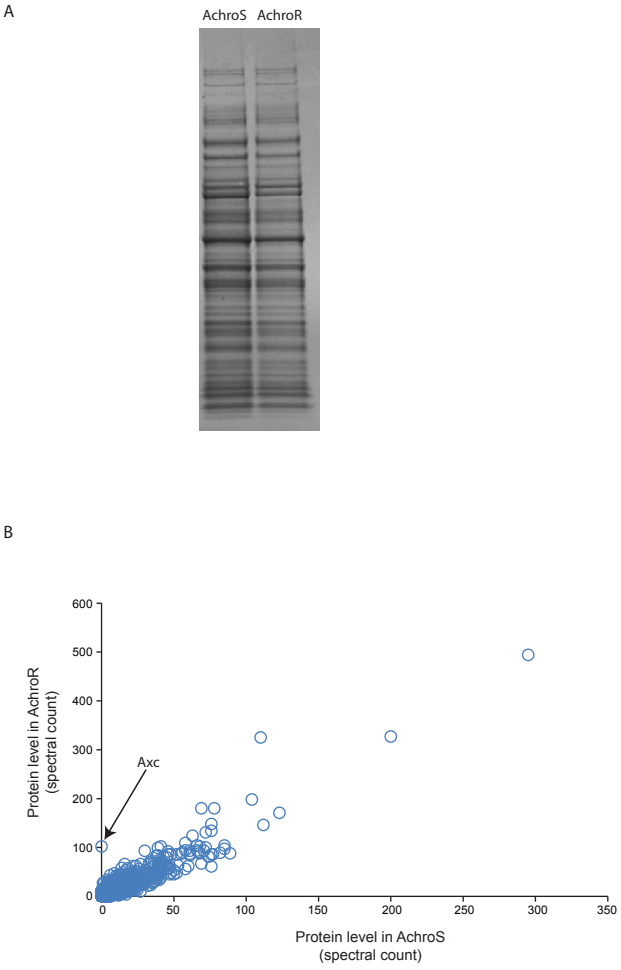
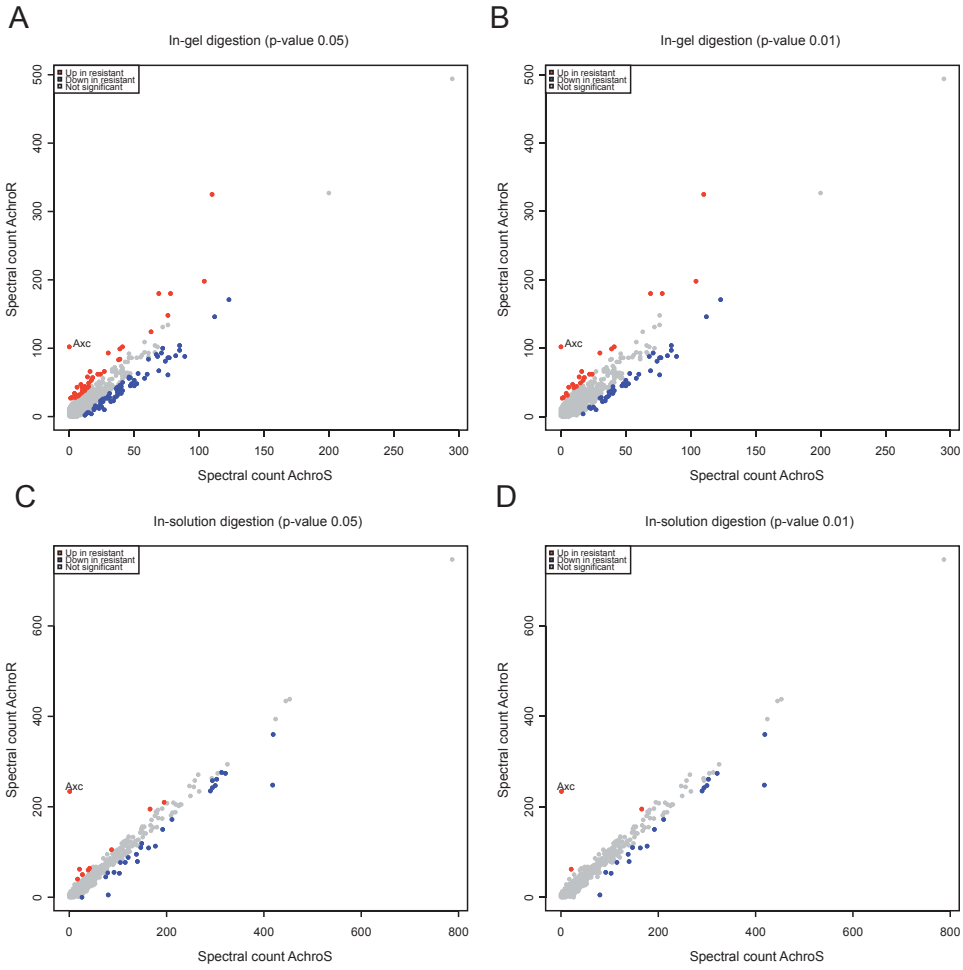
Supplemental data



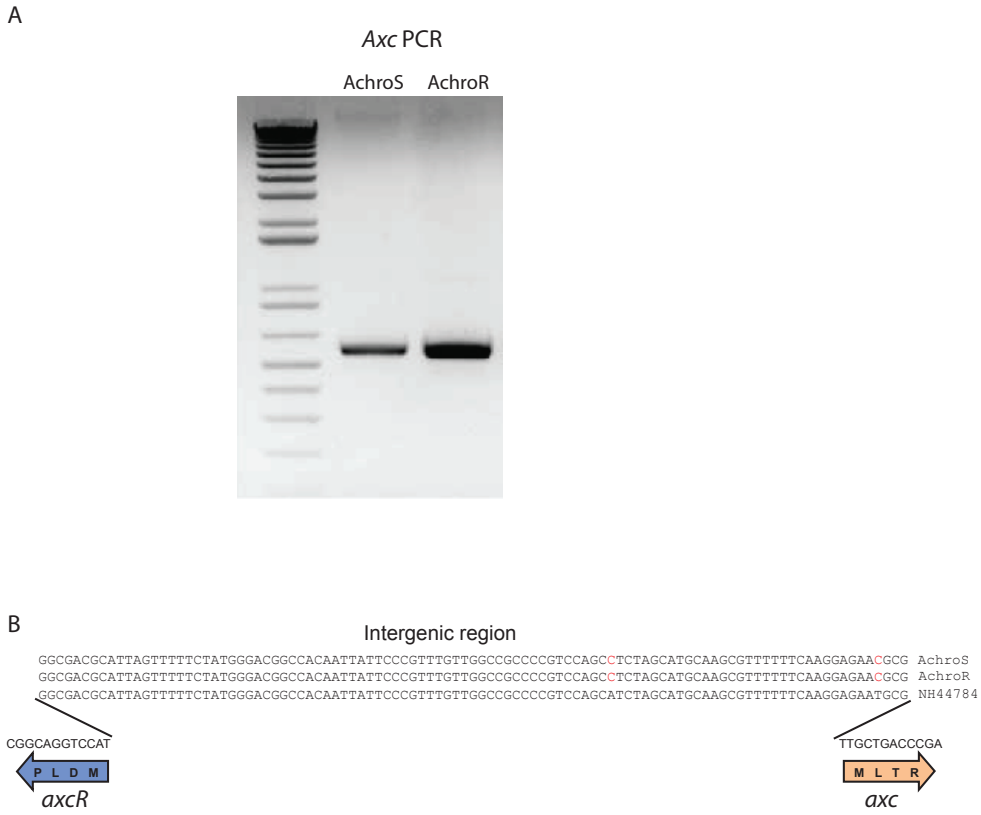
Supplemental figure S1: Quantitative proteomic analysis of the meropenem susceptible and resistant *Achromobacter xylosoxidans* clinical isolates.

A: Whole cell lysates from the two isolates (AchroS and AchroR, Table 1) were analyzed by SDS-PAGE and visualized by Coomassie staining. See supplemental figure S4 for a full gel picture, including MW markers.
B:Lanes from panel A were sliced into small bands and proteins were subsequently digested using trypsin. Following LC-MS/MS analyses and protein identification, spectral count analysis was used for relative protein quantification. Each dot represents the number of spectral counts observed per unique protein. Axc is the protein with the biggest difference in protein abundance between the resistant and susceptible isolate. In Table S2 and figure S2, the spectral counts and Qntdiff output can be found, respectively.



Supplemental figure S2: Analysis of differential proteins based on spectral counting using Qntdiff

The scatterplots display all quantified proteins using each protein's AchroS spectral count as x-coordinate and the corresponding ArchroR spectral count as y-coordinate, revealing a linear nature in the correlation between the spectral count values for each protein in both isolates. We devised a simple differential detection model that lumps all technical and biological variation into a null distribution for the perpendicular distances of proteins to a linear regression line through the data. If samples are similar enough, this method results in a p-value for the deviation from linearity of each protein. This method is implemented in our program Qntdiff. In figures A and B this method is applied to the in-gel digested data-set and shows differential proteins for p-value thresholds of 0.05 and 0.01, respectively. Figures C and D show the differential proteins selection in the in-solution digestion data-set for p-value thresholds of 0.05 and 0.01, respectively.

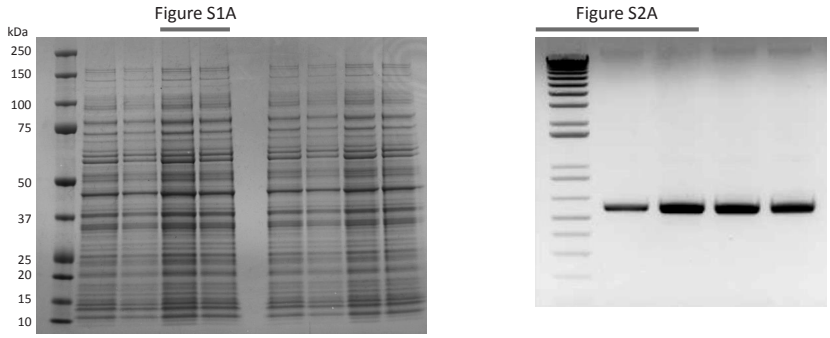


Supplemental figure S3: Presence of *axc* and sequence of its regulatory region in the meropenem resistant and susceptible clinical isolates

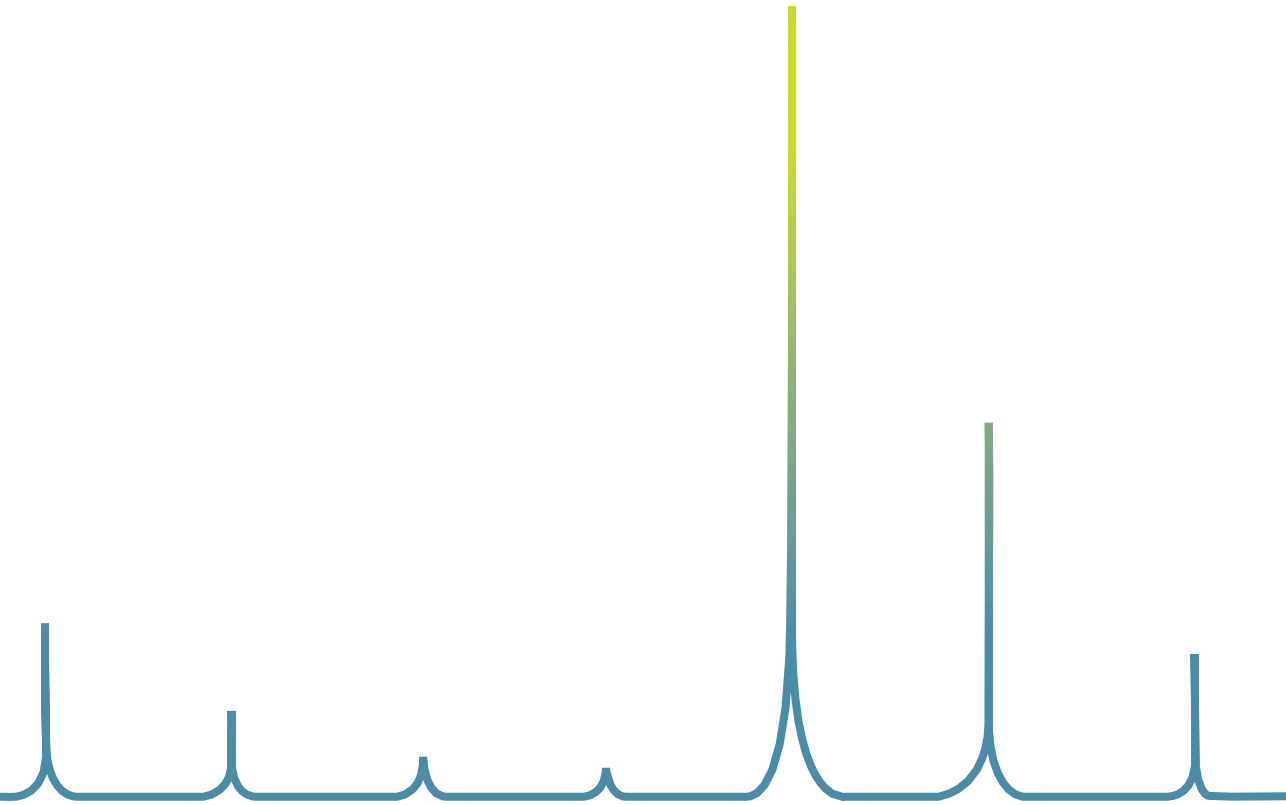
A: PCR using primers AxFor and AxRev (Table 3) showed that *axc* is present in both the meropenem resistant (AchroR) and meropenem susceptible (AchroS) clinical isolate. See Figure S4 for a full gel picture from which the cropped version shown is derived.

B: Sequence analysis showed that the intergenic region between *axc* and the gene encoding its putative repressor (*axcR*), were identical in AchroS and AchroR. At two positions (in red) this sequence differed from *A. xylosoxidans* strain NH44784-1996).

Full size gel pictures belonging to fig. S1A and S3A

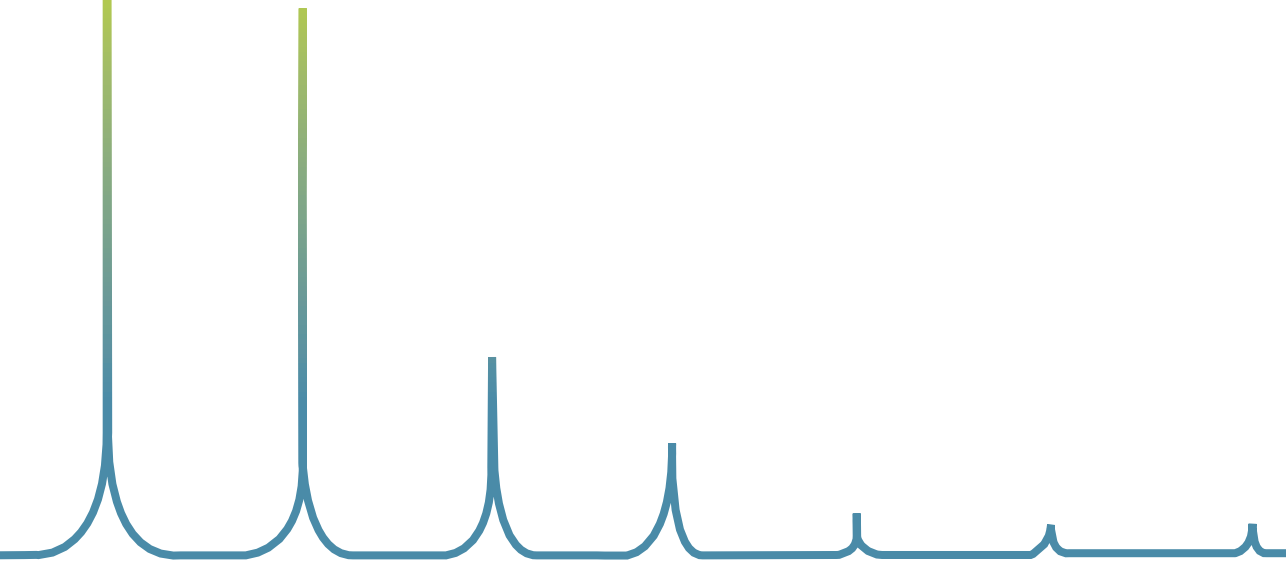


Supplemental figure S4: Full size gel pictures belonging to Fig. S1A and S3A



CHAPTER 6

Summary and discussion



Following the completion of many genome projects, the field of proteomics has undergone a surge in development alongside technological developments, mainly that of mass spectrometry[1,2]. The large-scale analysis of proteins by mass spectrometry is currently a key technology to study biological processes, to understand pathological processes and to identify disease-specific markers[3,4]. This thesis describes the development and application of such platforms for rapid and accurate identification of (multi-)drug resistant Gram-negative bacteria. Third generation cephalosporins, aminoglycosides and carbapenems are amongst the most prescribed antibiotics in hospitals. Resistance to these antibiotics is a major and rapidly growing problem worldwide[5,6]. Rapid and reliable detection of antibiotic-resistant bacteria, and the identification of novel resistance determinants, is important for patients care and to prevent spread of these multidrug resistant bacteria.

Chapter 2 details the development and application of a new platform to detect carbapenemases in (multi-)drug-resistant Gram-negative bacteria using capillary-electrophoresis mass spectrometry (CE-MS). This technological innovation uses a novel sheathless interface to hyphenate capillary electrophoresis with mass spectrometry. The potential advantages of this innovation result in an enhanced sensitivity and greater coverage of (hydrophilic) peptides in digested protein extracts of bacteria. We developed a straightforward sample preparation protocol, where bacterial lysis, protein solubilization and proteolytic digestion to peptide mixtures are all performed sequentially in a one tube. We found that the platform provides sufficient sensitivity to identify beta-lactamases in all analyzed bacterial extracts of lab strains. Subsequently, a proof-of-principle study was applied to a set of in-house characterized clinical isolates of Enterobacteriaceae containing OXA-48 (n = 17) and KPC (n = 10) carbapenemases. The results obtained with CE-MS were compared to the gold standard for carbapenemase (PCR). Furthermore, we compared the CE-MS analysis with two standard phenotypical assays, the modified Hodge test and a carbapenem degradation assay using MALDI-TOF MS. We found that the proteomic analysis was better able to establish the presence of carbapenemases, particularly those of the OXA-48 class. OXA-48 producers are known to be difficult to characterize phenotypically due to the range of susceptibility patterns[7].

This proteomic analysis also identified a number of extended spectrum beta-lactamases (ESBLs) in 19 of 27 clinical isolates. These were found without phenotypical or genomic foreknowledge of their presence, thereby emphasizing the broader suitability of the method provided that data analysis is performed using a comprehensive database. Overall, our study results demonstrated that peptide analysis can be used to identify ESBLs and carbapenemases. The total analysis takes 12-18 hours, mostly due to the need for tryptic digestion. Alternative sample preparation methods such as microwave assisted digestion can reduce this time further[8]. Alternatively, further improvements in top-down proteomics could

potentially result in the omission of the digestion step altogether, although technologically speaking, this seems to not be feasible on a short term.

A substantial improvement in the time-to-result is possible when the culturing step could be circumvented. Hence, we were interested to investigate the potential of a proteomic platform to identify ESBLs directly in blood cultures. Because ESBLs are a group of beta-lactamases which confer resistance to a wide array of beta-lactams, including third generation cephalosporins, this group is associated with high morbidity and mortality[9,10]. Accurate and early diagnosis of the presence of ESBLs is therefore critical to disease progression. Since the overall reliability and robustness of the CE-MS platform was not optimal and the background of blood derived proteins and matrix could be even more problematic, we decided to use a LC-MS/MS platform for these analyses (**chapter 3**). Blood culture media poses a significant challenge for mass spectrometric analysis and the sample preparation had to be optimized. In a prospective study in two university hospitals, all positive blood cultures of Gram-negative bacteria were examined by LC-MS/MS in addition to molecular and phenotypic techniques for recognition of ESBL-producing bacteria. In a period of four months, 170 *E. coli* positive blood cultures were collected, of which 22 contained an ESBL-producing bacterium. The LC-MS/MS-based method for characterization of the ESBL was correct in 95% of the cases. Especially for the CTX-M ESBLs (95% of the cases) the method performed excellent (100 %) and no false-positives were found in the non-ESBL producing positive blood cultures. These results were confirmed by molecular characterization of the corresponding genes.

Per analysis more proteins were identified by LC-MS/MS when compared with the CE-MS/MS study. The sequence coverage per identification was also generally higher. This better coverage resulted in classification of CTX-Ms into subgroups. In the cohort study, no carbapenemases were found since these resistance enzymes are very rare in The Netherlands. The use of a better mass spectrometer (with a faster cycling time and more sensitivity) could also potentially further enhance the sequence coverage which will aid in the discrimination between ESBL and non-ESBLs such as those present in the SHV class of beta-lactamase.

The beta-lactamases are only one class of enzymes involved in the resistance towards antibiotics. One of the initial goals of our research was also to develop a diagnostic platform for the detection of aminoglycoside resistance. This resistance is most commonly conferred due to the presence of aminoglycoside modifying enzymes, with the three major classes being aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs)[11]. To evaluate the application a proteomic platform to detect aminoglycoside modifying enzymes, we performed a number of pilot studies, using a laboratory strain expressing aminoglycoside 3'-phosphotransferase (APH(3')). However, both with CE-MS/MS and LC-MS/MS we were unable to detect their presence. This is probably associated with the low levels of these enzymes compared to

the beta-lactamases. Hence, we believe that a more sensitive mass spectrometry analysis with high sensitivity and faster cyling times (i.e. Orbitrap or triple-TOF instruments) and/or improved sample preparation workflows could potentially solve this issue.

If the analysis is focused on one or a few proteins only, selected-reaction monitoring (SRM) or multiple-reaction monitoring (MRM) provides a good alternative. SRM uses predefined pairings of mass spectrometric parent and product ions for identification and quantification of proteins of interest[12,13]. When a number of these different pairings are considered in the same analysis, this type of analysis is referred to as MRM. This means that the resulting analysis focuses on these targets exclusively, which results in enhanced sensitivity at the cost of a broader view. In a case such as the differentiation of a non-ESBL SHV from an ESBL-SHV, or the identification of aminoglycoside resistance, this could be a valuable approach. Typically these type of experiments are performed using a triple quadrupole mass spectrometer, where in the first quadrupole the parent mass is selected, in the second collision induced dissociation takes place to create fragments, while the third quadrupole filters the specific fragment ion of interest. The combination of a quadrupole with a high mass accuracy mass analyzer (e.g. Orbitrap or Tof) gives a similar option of targeted analysis, parallel reaction monitoring (PRM). Especially in combination with affinity purification, where antibodies are used to enrich for a specific protein of interest[14], such workflows provide better selectivity and sensitivity but require a significant investment for each protein of interest.

In addition to protein specific information, the proteomics data generated on the CE-MS/MS and LC-MS/MS platforms can also be used to type bacterial species for taxonomic purposes or epidemiological studies. Recent studies have used such datasets for bacterial typing including such species as *E. coli, S. aureus, P. aeruginosa, H. influenza*[15-20]. However, the analyses are quite laborious and time-consuming so we were interested in a more straightforward mass spectrometry method for bacterial typing. Obviously, the MALDI-ToF MS systems that have been introduced in the clinical microbiology practice would seem logic but attempts to use these platform for bacterial typing have only been reported in a limited number of studies for *E. coli*[21] and *K. pneumoniae*[22]. In general, studies show that the limited dynamic range and resolution of MALDI-TOF MS, results in spectra not containing sufficient information for reliable typing[23]. In **chapter 4** we explored the use of an ultrahigh resolution typing platform using MALDI-Fourier transform icon cyclotron resonance (FTICR) mass spectrometry. The platform preserves the speed of analysis and simplicity of sample preparation, i.e. the analysis of bacterial extracts spotted directly on the target plate. Following a pilot study to assess the spectral quality, a larger study was performed on a set of 18 well characterized *Pseudomonas aeruginosa* strains. This collection consisted of three clusters, as shown by amplified fragment length polymorphism (AFLP) analysis. The clusters were also phenotypically different, with one of the clusters containing all ciprofloxacin resistant strains. Using MALDI-FTICR MS, we were able to reproduce this clustering, but on a time scale similar to MALDI-TOF MS analysis for

species identification. We also performed clustering analysis using MALDI-TOF MS, but were not able to separate the strains into clusters. Hence, the MALDI-FTICR approach could be a valuable tool to screen the distribution and clonal spread of multi-resistant bacteria at local, national and international level. Interestingly, top-down analysis (for protein identification without proteolytic digestion) resulted in the identification of a specific protein isoform within the cluster of strains that were resistant to ciprofloxacin but further investigation is necessary to determine whether this protein can serve as a biomarker for ciprofloxacin resistance in *P. aeruginosa* or is involved in the resistance mechanism itself. Currently, we are optimizing this method for typing of *Clostridium difficile* but this scope can be broadened to other bacterial species. With straightforward sample preparation, high resolution profiling can be incorporated in parallel to existing species identification workflows in the laboratory.

Comparative studies on a proteome wide level are expanding our knowledge of the way bacteria interact with their environment to grow and survive. Proteome-wide studies of bacteria have been performed in species such as e.g. *Pseudomonas aeruginosa*[24,25], *E. coli*[26,27] and *K. pneumoniae*[28,29]. Such studies aim to use the role of protein expression to study functional and phenotypical changes in bacteria in response to outside stimuli. These changes may relate to the organisms metabolism and mobility in the host, its virulence or the response to antibiotics. For example, changes over time during infection can be monitored to study the real-time adaptation of bacteria[30]. Membrane permeability and associated antibiotic resistance on the proteomic level are of particular interest[31-36]. Potentially these studies can establish permeability thresholds where the phenotype switches from susceptible to resistant, which can be quantitatively determined. This opens up the possibility of establishing a new diagnostic platform. A different area of interest is the study of biofilms, both with regards to their development and often observed antibiotic resistance[37-42]. Biofilms can have varying degrees of complexity, depending on the number of species present and the environment of the biofilm in the host. They are known to play an important role in the accumulation and persistence of microorganisms and to be reservoirs of antibiotic resistance, but it is often not clear which underlying mechanisms result in these properties. The varying expression levels of proteins and especially the changes over time can provide insight into this, as it reflects the interaction between host and pathogen(s). Like with the exploration of the microbiome in general, proteomics is being used to develop insight on a broader level as opposed to the focus on single organisms, as protein analysis is uniquely suited to this and provides insight that alternative types of analysis can not.

Chapter 5 demonstrates the value of a comparative proteomic analysis to identify novel resistant determinants. The study was initiated following the isolation of two strains of *Achromobacter xylosoxidans* from an immunocompromised patient with a severe hospital-acquired pneumonia and sepsis. During meropenem treatment, the clinical isolates changed from a meropenem susceptible to a meropenem resistant phenotype. Proteomic analysis

revealed a novel beta-lactamase, Axc-1. Heterologous expression of Axc-1 in a susceptible *E. coli* strain increased the MIC for meropenem and imipenem eightfold. Axc-mediated hydrolysis of meropenem could be demonstrated using a colorimetric assay and ^{1}H-NMR. Both strains contained *axc* and its (putative) regulator *axcR*. Whole genome sequencing revealed one single nucleotide polymorphism (SNP) in *axyZ* in the resistant strain. AxyZ is the repressor of the *axyXY-oprZ*. This AxyZ isoform found in the meropenem resistant isolate (Axy_Gly29) results in higher expression of AxyY[43]. Hence, our data suggests that AxyZ is also a repressor of Axc but luciferase reporter assays should be used to confirm this. The study underscores the usefulness of proteomics as a discovery tool providing data that can directly influence patient care. In this particular case, the role of Axc-1 would have been missed when only a comparative genomic analysis would have been performed.

Clinical microbial mass spectrometry in context and outlook

Historically, proteomics in microbiology has been mostly used for basic research purposes. The main goal of this thesis was to explore and apply mass spectrometry based proteomics in clinical microbiology, especially focusing on antibiotic resistance. Many studies have investigated the proteomic changes as a result of antibiotic treatment[44-46]. With continuing studies and further technological advancements, we can expect many new insights into virulence and other mechanisms, revealing new therapeutic and diagnostic targets. A particular area where proteomics is uniquely positioned to provide new insights is that of post translational modifications (PTMs)[47-49]. These modifications are instrumental for cell signaling, enzyme regulation and protein interaction. While the role of PTMs has long been considered limited in bacteria, this view has changed[47]. All types of PTMs are observed in bacteria, from phosphorylation and acetylation which play a role in energy distribution and metabolism[50,51], to glycosylation and lipidation which influence adhesion, virulence and antibiotic resistance[47,52,53]. Interestingly, arginine phosphorylation, a rare modification in eukaryotic cells, seems to play a pivotal role in the control of protein degradation in bacteria, much like ubiquitin in eukaryotes[54].

Similarly, the past decade has seen an increased focus on the study of the gut microbiome as a whole, for insight into both host and pathogen biology[55,56]. Proteomic analysis is a key contributor in this field of research and will only grow to be more important[56-58]. Proteomics can provide insight into microbial activity and function but also monitor the interaction with the host and the effect of antibiotic therapy on the microbiome as a whole[59]. The gut microbiome plays a role in many diseases ranging from inflammatory diseases, cancer and metabolic disorders[57]. The enormous amount of data acquired by application of new mass spectrometric methods on the microbiome complicates the interpretation of metaproteomics and illustrates the need for dedicated bioinformatic expertise.

In addition to the MALDI-ToF based platforms for bacterial species identification, the use of new mass spectrometry-based applications for diagnostic purposes is currently implemented. For example, the ESBL and carbapenemase detection assays by MS are an interesting development. These are based on the principle of the detection of the hydrolysis of substrate antibiotics like cefotaxime/ceftazidime and imipenem/ertapenem, using mass spectrometry. The advantages of such an approach is the speed of analysis and relative low cost as these can be implemented into automated systems. Successes have been reported, such as ESBL detection in Gram-negatives[60,61] or the detection of KPC in *K. pneumoniae*[62]. Since these assays analyze the antibiotics and their conversion products, they are more sensitive to rapidly converting enzymes like KPC. We have found that enzymes with a lower activity such as OXA-48 are more difficult to reliably assess. Similar results have been reported in literature[7,63]. Further development of software tools to aid in the (quantitative) analysis of the mass spectrometric signals of the substrate and products can improve the applicability of the assay[64] and has already resulted in a better performance, comparable to phenotypic tests[65].

An alternative target for mass spectrometry analysis is the analysis of lipids. This type of analysis is useful as demonstrated in the context of the emergence of the mobilized colistin resistance genes (mcr-1 and mcr-2). Colistin is a polymyxin antibiotic, which binds to lipid A, and is frequently used as a last resort alternative in the case of extensive multidrug resistance. The discovery of *mcr-1* heralded the first plasmid mediated resistance factor to colistin and since 2015 it has been shown that it has spread extensively in bacteria enteric to humans and animals[66,67]. Mcr-1 modifies lipid A by catalyzing the transfer of phosphoethanolamine onto the lipid A, which severely reduces colistin binding[68]. Lipid A and its modifications can be identified using MALDI-ToF mass spectrometry[69]. Such screening of lipid A can be a valuable tool for the evaluation of the presence of Mcr-1[70,71]. The spread of different types of Mcr is being mapped rapidly, with up to six types currently having been identified[72]. This may have important implications for new diagnostic platforms to recognize colistin resistance.

The success of the MALDI-ToF MS-based platforms is for a large part due to the simplicity of the analysis, ease of data interpretation and robustness of the technique. Hence, it does not require a lot of training of personnel. On the other hand, the implementation of the platforms used in this thesis in the clinical microbiology lab will require more specific (technical) expertise and robust platforms. Therefore, it will take some more time before they may be as common as the MALDI-ToF MS-based platforms. For example, we showed that MALDI-FTICR MS-based typing offers a high resolution with sufficient discriminatory power for rapid bacterial typing. The major restriction of the technique is the prohibitive cost of acquisition and operation of the MALDI-FTICR MS platform. A feasible scenario is the use of regional centers such as academic hospitals that may already have similar equipment for research purposes, where rapid typing is available for inpatients or materials transferred from locations lacking these facilities.

Moreover, it is necessary to standardize the methodologies and data analysis used, to arrive at a method that can be implemented on a routine basis. While currently such analysis is prohibitively expensive, with increased standardization and natural decline of technological cost this could become feasible in the future. Summarizing, we can view the state of proteomics in the clinical microbiology as consisting of three main paths. In identification and typing, MALDI-TOF MS has effectively solved the species level identification and the past years have shown that new developments fail to comprehensively compete on time-to-result and throughput while remaining cost competitive. Subspecies and phenotypical typing remain as the areas of opportunity, however. While whole-genome sequencing will continue to be aspired to as the gold standard for comprehensive typing, we have demonstrated how proteomics can offer an alternative with superior speed and simplicity. With further developments proteomics can evolve to become a robust method for same-day subspecies and phenotypical typing with a broad applicability. In antibiotic susceptibility testing, proteomic approaches will not displace phenotypical testing as the standard methodology. There are however specific applications where protein analysis can provide a unique benefit over phenotypic and genotypic approaches. This is the case for enzymes with lower activity or in scenarios such as outbreaks where simultaneous phenotypical and epidemiological assessment is of interest. Studies into the role of post-translational modifications in response to antibiotic exposure may also yield new diagnostic targets that can only be assessed using proteomics. Lastly, proteomics will always be a powerful discovery tool. This not only includes the many mechanistic studies cited here, but also cases where a phenotype cannot be explained using standardized phenotypic and genotypic test, as demonstrated in our study on *Achromobacter xylosoxidans*. Proteomics and especially (semi-)quantitative analysis can provide a useful and unique insight into the function of any microorganism.

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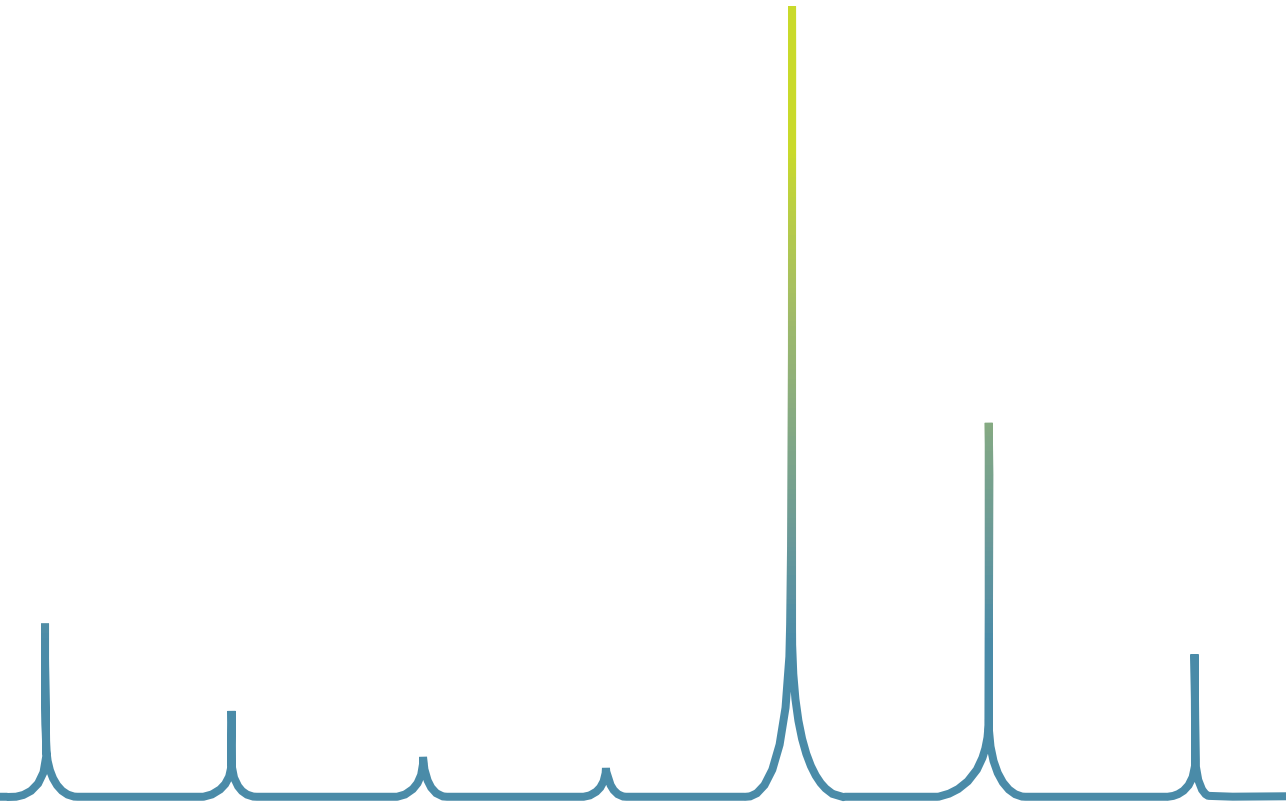

CHAPTER 7

Nederlandse samenvatting
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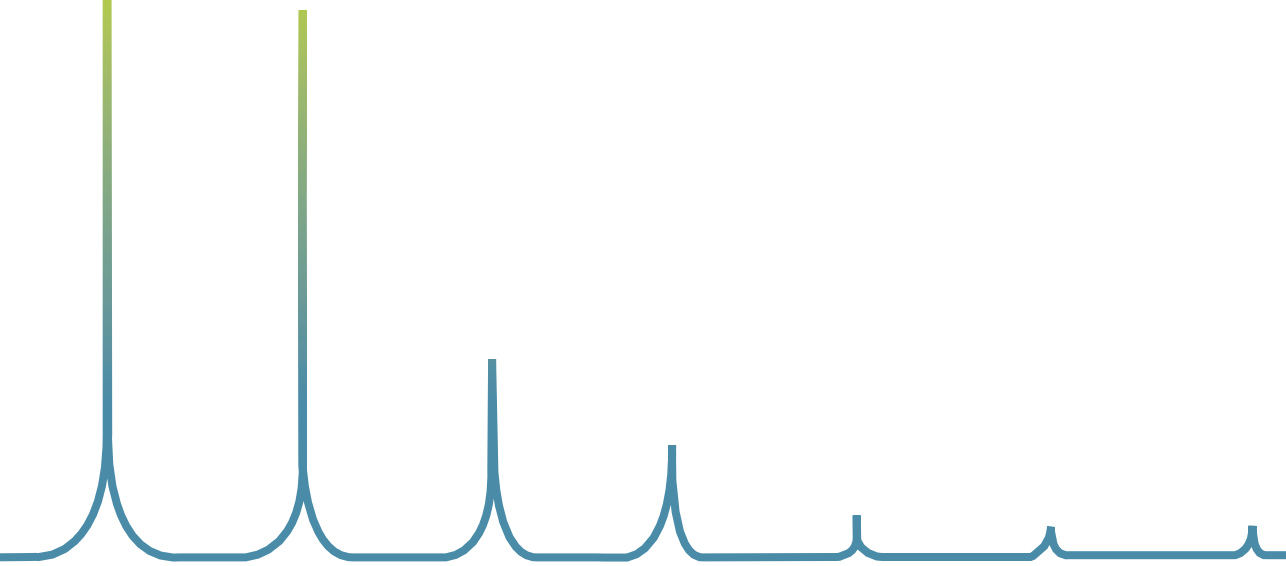

Nederlandse samenvatting

Bacteriële infectie en antibioticaresistentie

De ontdekking van antibiotica in de 20e eeuw heeft de strijd tegen infectieziekten volledig getransformeerd. De decennia na de Tweede Wereldoorlog worden gekenmerkt door een wapenwedloop tussen de introductie van nieuwe klassen van antibiotica en de daaropvolgende ontwikkeling en verspreiding van resistentie. Inmiddels is antibioticaresistentie een wijdverspreid probleem. In sommige gevallen is er zelfs sprake van zeer resistent micro-organismen (highly resistant microorganisms, HRMOs), waarbij in het geval van een infectie bij een patiënt de behandelopties zeer beperkt zijn. Niet alleen is de aanwezigheid van resistente bacteriën een risico voor de patiënt zelf, tevens kunnen de resistente bacteriën zich ook naar andere individuen verspreiden.

Er is een breed scala aan antibiotica dat gebruikt wordt voor de behandeling van bacteriële infecties. De antibiotica zijn op verschillende manieren te classificeren. Ten eerste wordt er onderscheid gemaakt op basis van de chemische structuur van het antibioticum zoals bijvoorbeeld beta-lactam antibiotica, aminoglycosiden, glycopeptiden en fluoroquinolonen. Ten tweede wordt er onderscheid gemaakt in de specificiteit van een antibioticum, dat wil zeggen of deze slechts tegen één of enkele soorten effectief is (nauw spectrum) of juist een grotere groep (wijd spectrum). Het behoeft geen betoog dat het juist identificeren van de bacteriële verwekker en het bijbehorende resistentieprofiel cruciaal zijn bij het succesvol bestrijden van infecties en het voorkomen van verdere verspreiding van resistente bacteriën.

Eiwit identificatie als analyse methode voor het vaststellen van antibioticaresistentie

Het antibioticaresistentie profiel van een bacterie is belangrijk voor het bepalen van de behandelingsstrategie. Fenotypische testen zijn hiervoor nog altijd de gouden standaard. De grenswaarde in milligram per liter waarbij een antibioticum de bacteriële groei tegengaat bepaalt of een middel gebruikt kan worden voor de behandeling van patiënten met een infectie. De minimale inhiberende concentratie (MIC, minimum inhibitory concentration) is de laagste concentratie waarbij een antibioticum effectief . Centrale instanties zoals de Clinical and Laboratory Standards Institute (CLSI) en European Committee on Antimicrobial Susceptibility Testing (EUCAST) maken richtlijnen die worden gebruikt om deze MICs te bepalen en de data te interpreteren. Deze fenotypische bepalingen voorspellen of een micro-organisme resistent is, maar geven in het algemeen geen inzicht in de onderliggende mechanismen die deze resistentie veroorzaken.

Er zijn meerdere manieren waarop een micro-organisme resistent kan worden. Dit kan door de netto opname van antimicrobiële stoffen te verlagen door opname te verlagen of uitscheiding te verhogen. Hierbij spelen o.a. porines en efflux pompen een belangrijke rol. Een andere mogelijkheid is het onschadelijk maken van de stof zelf, bijvoorbeeld door het

te modificeren of af te breken. Bekende voorbeelden hiervan zijn enzymen die beta-lactam verbindingen kunnen hydrolyseren (beta-lactamases, carbapenemases) en aminoglycoside modificerende eiwitten. Er is inmiddels een groot scala aan moleculaire methoden, voornamelijk PCR, die de aanwezigheid van deze resistentiegenen kunnen bepalen. In dit proefschrift hebben we echter gekeken of we met behulp van proteomics analyses met behulp van massaspectrometrie, de aanwezigheid van de corresponderen eiwitten snel en accuraat konden aantonen. Hierbij hebben we gekozen voor een strategie, bottom-up proteomics, waarbij eiwitten in eerste instantie worden opgeknipt tot kleinere peptiden met behulp van het enzym trypsine, waarna de peptiden met de massaspectrometer worden geanalyseerd. Met behulp van zoekalgoritmes worden de verkregen spectra gezocht tegen databases om tot peptide/eiwit identificaties te komen. Proteomics is inmiddels niet meer weg te denken in het biomedisch onderzoek. Het gebruiken van deze techniek voor diagnostiek van antibioticaresistentie is echter niet grondig verkend. In hoofdstuk 2 geven wij de eerste aanzet voor een mogelijk platform voor de directe identificatie van carbapenemases in Gram-negatieve bacteriën. Carbapenems worden in de meeste gevallen ingezet als een middel voor de behandeling van infecties met multiresistente bacteriën. Onze hypothese was dat carbapenem resistentie kan worden aangetoond door het direct identificeren van carbapenemases. Hiervoor hebben we een methode gebruikt waarbij capillaire elektroforese gekoppeld is gekoppeld aan massa spectrometrie (CE-MS). Naast de hoge scheidingscapaciteit van capillaire electroforese is het voordeel van deze scheidingstechniek ook zeer geschikt is voor hydrofiele peptiden. Na optimalisatie is de uiteindelijke methode getest met een set (n = 27) van klinische carbapenem resistente isolaten en een aantal negatieve controles. Deze resistente isolaten bestonden uit een groep de oxacillinase-48 (OXA-48) achtige (n = 17) en *Klebsiella pneumoniae* Carbapenemases (KPC, n = 10).Ter vergelijking werden er ook een fenotypische- en een PCR analyse uitgevoerd. In alle carbapenem-resistente isolaten konden we met CE-MS het carbapenemase aantonen. De methode werkte ook goed om een enkele bacteriekolonie. De identificaties kwamen volledig overeen met fenotypische en PCR gebaseerde karakterisering van de carbapenemases. De OXA-48 carbapenemases staan bekend als fenotypisch lastig te bepalen carbapenemases omdat ze het carbapenem langzaam omzetten. Naast de verwachte carbapenemases weren er ook meerdere extended-spectrum beta-lactamases (ESBLs) gevonden. Dit toont aan dat de methode ook breder ingezet kan worden voor de evaluatie van meerdere typen antibioticaresistentie.

Het succesvol aantonen van carbapenemases in diverse Gram-negatieve klinische isolaten vormde de basis voor een breder proteomics platform. Resistentie tegen derde generatie cefalosporines middels extended-spectrum beta-lactamases (ESBLs) is een veel voorkomende antibioticaresistentie. In hoofdstuk 3 hebben we een methode ontwikkeld voor de analyse van ESBLs direct in positieve bloedkweken. Vanuit een proteomics oogpunt heeft een bloedkweekfles een complexere matrix dan een voedingsbodem

of bouillonkweek. Dit stelt hogere eisen aan de monstervoorbewerking en de algehele robuustheid van de analyse methode. Voor dit onderzoek hebben we gebruik gemaakt van een andere scheidingsmethode, namelijk vloeistof-chromatografie (liquid chromatography, LC) gekoppeld met massaspecteomtrie. Na meerdere opties onderzocht te hebben, is er gekozen voor een eenvoudige voorbewerking (gebruikmakend van saponine), waarbij de extracten werden verwerkt tot peptide mengsels die geanalyseerd werden met LC-MS/MS. Behalve de hogere robuustheid zou deze methode gevoeliger zijn omdat met deze methode er een grotere hoeveelheid monster geanalyseerd kan worden vergeleken met CE. Met deze nieuwe methode is er een prospectieve studie in twee academische centra opgezet, waarbij gedurende vier maanden alle positieve bloedkweken (n = 170) met Escherichia coli (n = 125) of Klebsiella pneumoniae (n = 45) verzameld werden. Van deze 170 kweken bleken er 22 ESBL positieve isolaten op basis van fenotypische analyse. Deze ESBL positieve bloedkweken zijn samen met 44 willekeurig geselecteerde negatieve controles (bloedkweken met niet-ESBL producerende bacteriën) geanalyseerd met LC-MS/MS. Van de gekweekte isolaten is ook een PCR analyse voor relevante ESBLs uitgevoerd. Met behulp van LC-MS/MS zijn de aanwezige ESBLs correct geïdentificeerd in 95 % van de bloedkweken, zonder vals positieve resultaten in de negatieve controles. Voor de familie van CTX-M ESBLs, welke 95% van de aanwezige ESBLs betrof, was er in alle gevallen een juiste identificatie en er kon ook onderscheid tussen groep 1 en groep 9 CTX-M gemaakt worden. Tijdens de studie zijn er geen carbapenemases aangetroffen. Resumerend toont de studie de geschiktheid van een proteomics platform voor vroege herkenning van ESBL poducerende Gram-negatieve bacteriën in positieve bloedkweken.

Het identificeren en typeren van bacteriën op basis van het proteoom

Identificatie van bacteriën wordt traditioneel uitgevoerd door middel van Gram-kleuring, kweekmethoden en biochemische analyses. Meer recent gebeurt dit door eiwitanalyse met behulp van matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) massaspectrometrie (MS). Met behulp van deze techniek kan een profiel van eiwitten/peptiden bepaald worden dat karakteristiek is voor de aanwezige bacteriesoort. Het voordeel ten opzichte van de traditionele methoden is dat de analysemethode relatief eenvoudig en goedkoop is, waardoor het minder tijd vereist en sneller is. Dit heeft erin geresulteerd dat binnen enkele jaren MALDI-TOF MS instrumenten op vrijwel alle microbiologische laboratoria geïntroduceerd zijn.

Voor het onderzoeken van de verwantschap van bacteriën binnen een soort, en het herkennen van hypervirulente typen, zijn er een aantal typeringstechnieken beschikbaar. Deze technieken worden ook gebruikt bij een uitbraak om verspreiding vast te stellen en een mogelijke bron te ontdekken. Typering kan op basis van een fenotypische eigenschap zoals bijvoorbeeld antibioticaresistentie of op basis van genetische informatie (genotypering). Het ontbreekt momenteel echter aan een eenvoudige techniek met voldoende onderscheidend

vermogen die typering op een korte tijdschaal toestaat. De gebruikelijke moleculaire typeringsmethoden (bijvoorbeeld met PCR-ribotypering, whole genome MultiLocus Seqeunce Typing (wgMLST)) of core-genome MLST (cgMLST) zijn veelal traag, kostbaar en vereisen een complexe data analyse. Er zijn meerdere pogingen gedaan om eiwit profielen die gebruikt worden voor de identificatie van bacteriesoorten in te zetten voor typering. Hoewel meerdere studies claimen dat MALDI-TOF MS gebruikt kan worden voor de typering van diverse soorten Gram-negatieve bacteriën, zijn deze studies moeilijk te reproduceren in andere laboratoria. MALDI-TOF MS als een platform is niet krachtig genoeg en heeft een gebrek aan onderscheidend vermogen om robuuste typering te faciliteren. Er is echter een alternatieve analysemethode die met dezelfde simpele voorbewerking en op dezelfde tijdschaal hoogwaardigere data genereerd die mogelijk voor typering gebruikt kan worden. Deze techniek gebruikt dezelfde ionisatie methode (MALDI), gekoppeld aan een hoge resolutie analyse te weten Fourier transform ion cyclotron resonance (FTICR). In hoofdstuk 4 hebben wij deze methode geoptimaliseerd en getest op toepasbaarheid voor de typering van Pseudomonas aeruginosa stammen. Hiervoor is een set van 18 klinische isolaten uit verschillende ziekenhuizen gebruikt. Met behulp van MALDI-FTICR was het mogelijk om hierin drie clusters te onderscheiden. Onafhankelijke moleculaire en fenotypische analyse bevestigden dat deze set uit drie clusters bestond. In één van de drie clusters vonden we resistentie tegen het antbioticum ciprofloxacine. Kortom, de studie toonde aan dat MALDI-FTICR MS snelle typering van bacteriën mogelijk maakt. Verdere ontwikkeling zal moeten resulteren in een robuustere methode en de verwachting is dat de methode met minimale aanpassingen toepasbaar is voor een verscheidenheid aan bacteriesoorten. Aangezien de aanschaf- en gebruikskosten voor dit apparaat vele malen hoger zijn, zijn deze instrumenten echter nog lang niet overal beschikbaar.

Proteomics als discovery tool voor nieuwe resistentiefactoren

Naast het identificeren van bekende eiwitten die resistentie kunnen veroorzaken is proteomics ook zeer geschikt voor onderzoek naar nieuwe resistentiefactoren. In hoofdstuk 5 is een studie uitgevoerd waarbij een carbapenem (meropenem) gevoelige en resistente stam van Achromobacter xylosoxidans met elkaar zijn vergeleken. Deze klinische isolaten waren afkomstig van een patiënt waarbij in een zeer korte tijd, tijdens de behandeling met meropenem, resistentie was ontstaan. Om het onderliggende mechanisme uit te zoeken is in eerste instantie een vergelijkende semi-kwantitatieve proteomics analyse uitgevoerd. Hieruit bleek dat één eiwit, dat wij Axc hebben genoemd, veel aanwezig was in de resistente stam maar niet in de gevoelige stam. Dit eiwit stond geannoteerd als klasse A beta-lactamase op basis waarvan de carbapenemase onduidelijk was. Om de carbapenemase activiteit van Axc te onderzoeken is het recombinant tot expressie gebracht in E. coli. Met behulp van fenotypische testen konden we op die manier laten zien dat deze cellen inderdaad hoger MICs voor imipenem en meropenem hadden. Vervolgens konden we met behulp van een colorimetrische assay en 1H-NMR direct Axc-gemedieerde hydrolyse van imipenem

aantonen. Opvallend genoeg toonden moleculaire tests aan dat het axc-gen in beide *A. xylosoxidans* stammen identiek was. Daarnaast bleek na whole genome sequencen dat beide stammen, op één single nucleotide polymorphism na, genetisch identiek waren. Dit polymorfisme werd gevonden in een repressor waarvan door andere onderzoekers in een eerder stadium is aangetoond dat de aminozuurverandering die we in de resistente stam vonden, de activiteit van de repressor verlaagt. Kortom, op basis van onze studie konden we de nieuwe hypothese opstellen dat deze repressor ook de expressie van axc reguleert. In het algemeen bleek onze studie een mooi voorbeeld van de unieke mogelijkheden van proteomics, omdat de rol van Axc in de resistentie tegen meropenem niet zou zijn gevonden als alleen whole genome sequencing zou zijn uitgevoerd.

Perspectief

Het onderzoek zoals beschreven in dit proefschrift laat zien dat er naast de MALDI-TOF MS, dat reeds algemeen geaccepteerd, vele andere massaspectrometrische analyses van eiwitten mogelijk zijn die meer specifieke informatie kunnen verschaffen die interessant zijn voor de klinische microbiologische diagnostiek. Deze methoden kunnen niet alleen essentieel zijn voor onderzoek aan nieuwe resistentiefactoren, zoals onze studie aan A. xylosoxidans aantoont, maar mogelijk ook in de richting van diagnostische platforms voor typering en bepaling van resistentie, waarvoor we hier een aanzet hebben gegeven. Echter, verdere ontwikkelingen met betrekking tot de robuustheid en gebruikersvriendelijkheid van deze platforms zijn nodig voordat ze ook wat dit betreft dezelfde standaard als MALDI-TOF MS zouden kunnen worden.

List of publications

Sorted by year, this thesis concerns 2014 onwards

Frank Fleurbaaij, Alex A. Henneman, Jeroen Corver, Cornelis W. Knetsch, Wiep Klaas Smits, Sjoerd T. Nauta, Martin Giera, Irina Dragan, Nitin Kumar, Trevor D. Lawley, Aswin Verhoeven, Hans C. van Leeuwen, Ed J. Kuijper, Paul J. Hensbergen
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Curriculum vitae

Frank Fleurbaaij was born on November 9th, 1988 in Amsterdam, The Netherlands. In 2006 he graduated from het Willem de Zwijger College in Bussum and started his studies in Chemistry at the VU University in Amsterdam. He obtained his Bachelor's degree in 2010 after completing a research project on a derivatization strategy for screening endogenous biomarkers of free radical damage. Next he enrolled in the joint Analytical Chemistry master program of the VU University and the University of Amsterdam (UVA). At the VU University he was responsible for a project developing a high-resolution metabolic profiling method for the rapid and comprehensive screening of histamine H4 receptor ligands for G protein-coupled receptors, at the BioMolecular Analysis group at the VU University, under supervision of Dr. Jeroen Kool. This project was nominated for the KNCV Golden Master Award 2011 and formed the basis of a publication in the Journal of Chromatography A. During this time he also spent four months at King's College at the Analytical & Environmental Science Division led by Dr. Norman Smith, developing and applying monolithic stationary phases in capillary columns for bioanalysis purposes. After obtaining the Master's degree in the summer of 2012, he started as a PhD candidate at the Department of Medical Microbiology in the Leiden University Medical Center. This project was under the supervision of prof. dr. Ed Kuijper and dr. Hans van Leeuwen and in close collaboration with dr. Paul Hensbergen of the Center for Proteomics and Metabolomics. This work, which is largely described in this thesis, concerns the development of novel applications of proteomics analysis using mass spectrometry in clinical microbiology. The scope of this research ranges from antibiotic resistance determination to bacterial typing using high resolution mass spectrometry. The collaborative nature of the research is signified by collaborations with various research groups, both domestic and abroad. Parts of this work have been presented at a number of (inter)national congresses including Microscale Bioseparations (2013, 2014), the European Congress of Clinical Microbiology and Infectious Disease (2014, 2016), Human Proteome World Congress (2015), Dutch society for medical microbiology (2016) and the Dutch society for mass spectrometry (2016). Following on from his time at the university hospital, he spent a year working in the petrochemical industry at Shell Pernis in Rotterdam (2017). Currently Frank is employed at the Netherlands Forensic Institute (NFI) where he is part of the Gunshot residue analysis team at the Division of Chemical and Physical Traces.

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