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Carbapenem resistance in *Acinetobacter pittii* mediated by metallo- β -lactamases

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Die dieser Arbeit zugrunde liegenden Isolate von *Acinetobacter pittii*-Bakterien wurden im Nationalen Referenzzentrum für gramnegative Krankenhauserreger der Ruhr-Universität Bochum gesammelt und mir für diese Arbeit von Herrn Dr. Kaase zur Verfügung gestellt.

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Abbreviation index

AB-PBRT	<i>A. baumannii</i> PCR-based replicon typing
ACB complex	<i>Acinetobacter calcoaceticus</i> – <i>Acinetobacter baumannii</i> complex
ACT	AmpC-type β -lactamase
ADC	<i>Acinetobacter</i> derived cephalosporinase
AIM	Adelaide imipenemase
AMR	antimicrobial resistance
ARG	antibiotic resistance gene
BcII	<i>Bacillus cereus</i> metallo- β -lactamase
BJP	<i>Bradyrhizobium japonicum</i> metallo- β -lactamase
bp	base pairs
CAR	<i>Erwinia carotovora</i> metallo- β -lactamase
CAU	<i>Caulobacter crescentus</i> metallo- β -lactamase
cgMLST	core genome multi locus sequence typing
CphA	<i>Aeromonas hydrophilia</i> carbapenemase
CTX-M	cefotaxime Munich β -lactamase
dcgMLST	distributed core genome multi locus sequence typing
DIM	Dutch imipenemase
FIM	Florence imipenemase
GES	Guiana extended-spectrum β -lactamase
GIM	German imipenemase
GTA	gene transfer agent
HGT	horizontal gene transfer
ICE	integrative conjugative elements
ICU	intensive care unit
IMP	imipenemase
IR	inverted repeat
IS	insertion sequence
kbp	kilo base pairs
KHM	Kyorin Health Science metallo- β -lactamase
KPC	<i>Klebsiella pneumoniae</i> carbapenemases
MBL	metallo- β -lactamase
MDR	multi-drug resistant
ME	mobilizable element
MPF	mating pair formation
NDM	New-Delhi metallo- β -lactamase

OMV	outer membrane vesicle
orf	open reading frame
OXA	oxacillinase
PBP	penicillin-binding protein
PDR	pan-drug resistant
POM	<i>Pseudomonas otitidis</i> metallo- β -lactamase
SFH	<i>Serratia fonticola</i> hydrolase
SHV	sulfhydryl reagent variable β -lactamase
SIM	Seoul imipenemase
sp.	Species
SMB	<i>Serratia marcescens</i> metallo- β -lactamase
SPM	São Paulo metallo- β -lactamase
Ssr	site specific recombinase
T4SS	type 4 secretion system
TEM	Temoniera β -lactamase
THIN	<i>Jathinobacterium lividum</i> metallo- β -lactamase
tIS	transporter insertion sequence
TMB	Tripoli metallo- β -lactamase
Tn	transposon
Tnp	transposase (protein)
VIM	Verona integron-encoded metallo- β -lactamase
WHO	World Health Organisation
XDR	extensively drug resistant
Zn	zinc

1. Summary

The genus *Acinetobacter* comprises a heterogeneous group of bacteria, some of which are environmentally occurring species, but also includes clinically relevant pathogenic species. Nosocomial infections are mostly caused by *Acinetobacter baumannii*, followed by other members of the *A. baumannii* group, such as *A. pittii* or *A. nosocomialis*. *Acinetobacter* species other than those belonging to the *A. baumannii* group are rarely originators of nosocomial infections.

Members of the genus *Acinetobacter* have shown an elevated pathogenic potential through their ability to form biofilms alongside an increased desiccation tolerance, both of which allow them to survive in the hospital environment. Although *A. baumannii* is still the most prevalent species, *A. pittii* has become more frequent throughout the years. Infections target mostly chronically ill, multimorbid or ventilated patients in ICU settings, while community-acquired cases are rare. The most common infection caused by members of the *A. baumannii* group is pneumonia, especially in ventilator-associated settings. Other potential foci include nervous system, soft tissue and bones as well as blood stream and the urinary tract.

Members of the *Acinetobacter* genus are inclined to develop antibiotic resistance quickly. This ability is often mediated via their ability to mutate, and up-regulate intrinsic antibiotic resistance mechanisms as well as their propensity to acquire resistance genes. The latter is founded in the ability for horizontal gene transfer, which is an umbrella term, denominating different mechanisms with which bacteria can garner genetic material, either from other bacteria or the environment. The most researched mechanism in *Acinetobacter* is conjugation, in which plasmids (circular strands of DNA) or integrative conjugative elements (typically chromosomally encoded elements, that can be excised) are transmitted in between two bacterial cells via a mating complex through direct cell contact. *Acinetobacter* species display a tremendous genome plasticity induced by the presence of mobile genetic elements such as insertion sequences, integrons and transposons, which are the main contributors to intracellular mobility. Insertion sequences and transposons are DNA elements that can translocate between the same or different DNA molecules, which makes exchange of genetic material between chromosomal DNA and a putatively mobilizable plasmid possible. Integrons harbour a gene encoding for an integrase, that can garner gene cassettes, often bearing resistance genes.

In *Acinetobacter* most MBLs have been shown to be associated with either an integron or transposons formed by insertion sequences. The high prevalence of mobile genetic elements leads to an increased number of multi-drug resistant isolates. Resistance to carbapenems is of particular concern, as they comprise the first-line treatment of *Acinetobacter* infections.

Resistance to β -lactams in *Acinetobacter* is generally mediated by the presence of β -lactamases, which can be grouped according to their amino-acid sequence into Ambler classes A to D. *Acinetobacter* produce two chromosomally encoded β -lactamases: an Ambler class C cephalosporinase (*bla*_{ADC}) and an Ambler class D oxacillinase (*bla*_{OXA}). Acquired resistance to β -lactams is often mediated by upregulation of *bla*_{ADC}, acquired class D oxacillinases or class B metallo- β -lactamases (MBL). Most frequent acquired MBLs include IMP, NDM or VIM.

As studies on carbapenem resistance in *Acinetobacter* spp. other than *A. baumannii* have been scarce, our aim was to investigate carbapenem resistance mediated through the presence of MBLs in clinical *A. pittii* isolates and characterize MBL-encoding plasmids when the resistance genes were found to be plasmid-borne.

Our study examined seventeen carbapenem-resistant *A. pittii* isolates from Germany, harbouring an MBL, which were collected between 2010 and 2015. MBL-encoding resistance genes identified in the isolates through whole genome sequencing were *bla*_{NDM-1}, *bla*_{GIM-1}, *bla*_{VIM-2} and *bla*_{VIM-4}. cgMLST showed that, while 3 isolates were most probably part of a singular outbreak, the other fourteen isolates were epidemiologically unrelated. The genetic platform of the MBLs was assessed using S1 pulsed field gel electrophoresis and southern blot. Ten isolates harboured a plasmid-encoded MBL (all isolates harbouring *bla*_{GIM-1}, *bla*_{VIM-2} and three of the isolates harbouring *bla*_{NDM-1}), while in seven isolates MBLs were shown to be chromosomally encoded (all isolates harbouring *bla*_{VIM-4} and four of the isolates harbouring *bla*_{NDM-1}). Plasmid mapping was conducted using hybrid assemblies via Unicycler of short- and long-read sequencing reads using both MiSeq and MinION platforms. Conjugation into *E. coli* and *A. baumannii* was tested using broth mating. Conjugation to *E. coli* was unsuccessful, while *bla*_{NDM-1}-encoding plasmids and four out of five *bla*_{GIM-1}-encoding plasmids were shown to be conjugative into *A. baumannii*. Despite isolates being clinically unrelated all plasmids showed high similarity. The plasmid backbones had identities of > 99 %, only significantly differing in the MBL-encoding area. The *bla*_{NDM-1}-harbouring plasmids were highly similar to other plasmids from *Acinetobacter* isolates worldwide, especially in Asia and the Americas, while the *bla*_{VIM-2}- and *bla*_{GIM-1}-encoding plasmids have not been described before. *bla*_{VIM-2} and *bla*_{GIM-1} were shown to be integrated into an array of resistance genes flanked by an integrase (*Int11-like*), which showed similarities to the *TnAs3* transposon. We thus hypothesized that the *bla*_{VIM-2}- and *bla*_{GIM-1}-encoding plasmids are variants of the original *bla*_{NDM-1}-encoding plasmid, that have acquired an integrase, possibly through action of a transposon, which was consecutively responsible for integrating resistance gene-bearing gene cassettes into the plasmid. These data show the occurrence of a new plasmid variant of a previously recognised

epidemic plasmid. While this other plasmid has been identified in isolates worldwide, this study is the first to describe these plasmid variants with MBLs other than *bla*_{NDM-1}. This finding could hint at the possibility that these new plasmid variants originate in Germany. Further research into MBL-encoding *A. pittii* in other European countries could deliver a snapshot of the current distribution of resistance patterns in *Acinetobacter* in Europe and elucidate the prevalence of plasmid variants of the original *bla*_{NDM-1}-encoding plasmid.

We should also recognize that, firstly, because of the extraordinary genome plasticity in *Acinetobacter* species, and the inter-species compatibility of mobile genetic elements, future comparative plasmid analysis should look beyond the borders of one species. Secondly, plasmid analysis, especially analysis of the resistome, should be conducted independently from the plasmid-host, as similar plasmids appear in unrelated host bacteria.

2. Zusammenfassung

Die Gattung *Acinetobacter* beinhaltet eine heterogene Gruppe von Bakterien, von denen einige lediglich als Umweltkeime anzusehen, andere hingegen von klinischer Relevanz sind. Nosokomiale Infektionen werden vorwiegend von *Acinetobacter baumannii* hervorgerufen, gefolgt von anderen Mitgliedern der *A. baumannii* Gruppe wie beispielsweise *A. pittii* oder *A. nosocomialis*. Andere *Acinetobacter* Spezies als jene, die der *A. baumannii* Gruppe angehören sind hingegen selten die Verursacher von nosokomialen Infektionen.

Spezies aus der Gattung *Acinetobacter* haben durch ihre Fähigkeit Biofilme auszubilden ein ausgeprägtes pathogenes Potential gezeigt, welches ihnen neben einer ausgeprägten Toleranz gegenüber Austrocknung ein Überleben in einer klinischen Umgebung erlaubt. Obwohl *A. baumannii* die Spezies mit der höchsten Prävalenz ist, ist das Auftreten von *A. pittii* im Verlauf der Jahre deutlich häufiger geworden. Infektionen betreffen am häufigsten chronisch erkrankte und multimorbide sowie beatmete Patient:innen in intensivstationären Settings, wohingegen ambulant erworbene Infektionen selten sind. Die häufigste Infektion, die durch Spezies aus der *A. baumannii* Gruppe verursacht wird ist die Pneumonie, insbesondere bei endotracheal beatmeten Patienten. Zu anderen möglichen Infektfoki zählen das Nervensystem, Weichteilgewebe, Knochen ebenso wie die Blutbahn und die Harnwege.

Spezies der Gattung *Acinetobacter* neigen zu einer raschen Resistenzentwicklung gegen Antinfektiva. Dieses Potential wird durch ihre Fähigkeit zur Mutation und zur Hochregulation intrinsisch vorliegender Resistenzmechanismen begünstigt, ebenso wie durch ihre Fähigkeit, rasch Resistenzgene aufnehmen zu können. Letzteres ist in der Fähigkeit zum horizontalen Gentransfer begründet. Hierbei handelt es sich um einen Sammelbegriff, der verschiedene Mechanismen zusammenfasst, durch die Bakterien genetisches Material entweder von anderen Bakterien oder aus der Umwelt erwerben können. Hierbei ist die Konjugation der bei *Acinetobacter* am meisten erforschte Mechanismus, bei dem Plasmide (zirkuläre DNA-Elemente) oder integrative konjugative Elemente (typischerweise chromosomale DNA-Fragmente, die aus dem Chromosom herausgelöst werden können) zwischen zwei Bakterien mittels direktem Zellkontakt transferiert werden können. *Acinetobacter* zeigen ebenso eine außergewöhnliche Genomplastizität, welche durch das Vorliegen von mobilen genetischen Elementen wie Insertionselementen, Integrans und Transposons hervorgerufen wird, die als Hauptbeteiligten an intrazellulärer Mobilität fungieren. Insertionselemente und Transposons sind DNA-Elemente, die sich zwischen demselben oder unterschiedlichen DNA-Molekülen mobilisieren können, was den Austausch von genetischem Material zwischen dem Chromosom und gegebenenfalls mobilisierbaren Plasmiden möglich macht. Integrans

hingegen sind DNA-Sequenzen, die für eine Integrase kodieren, die oftmals Resistenzgen-tragende Genkassetten in die DNA integrieren kann.

In *Acinetobacter* waren bislang die meisten MBLs entweder mit Integrans oder durch Insertionselemente gebildeten Transposons assoziiert. Die hohe Prävalenz von mobilen genetischen Elementen führt zu einer hohen Zahl an multiresistenten Isolaten. Hierbei ist die Resistenz gegenüber Carbapenemen besonders besorgniserregend, da diese die Erstlinientherapie gegen *Acinetobacter* Infektionen darstellen. Die Resistenz gegenüber β -Laktamantibiotika ist bei *Acinetobacter* in der Regel durch β -Laktamasen bedingt, die anhand ihrer Aminosäuresequenz in die Ambler-Klassen A bis D gruppiert werden können. *Acinetobacter* produzieren zwei chromosomal kodierte β -Laktamasen, eine Cephalosporinase der Ambler-Klasse B (*bla_{ADC}*) und eine Oxacillinase der Ambler-Klasse D (*bla_{oxA}*). Erworbene Resistenz gegenüber β -Laktamantibiotika wird oft durch die Hochregulierung von *bla_{ADC}* oder durch erworbene Oxacillinasen (Klasse D) oder Metallo- β -Laktamasen (MBL) der Klasse B verursacht. Als häufigste erworbene MBLs sind IMP, NDM oder VIM zu nennen.

Da die Forschung bezüglich Carbapenem-Resistenz in anderen *Acinetobacter* Spezies abgesehen von *A. baumannii* bislang lückenhaft ist, war es unser Ziel, Carbapenem-Resistenz durch MBL in *A. pittii* Isolaten zu untersuchen und hierbei entdeckte MBL-kodierende Plasmide vollständig zu charakterisieren.

Unsere Studie untersuchte siebzehn Carbapenem-resistente *A. pittii* Isolate aus Deutschland, die eine MBL kodierten und zwischen 2010 und 2015 isoliert wurden. MBL-kodierende Resistenzgene, die in den Stämmen mittels whole genome sequencing identifiziert werden konnten waren *bla_{NDM-1}*, *bla_{GIM-1}*, *bla_{VIM-2}* and *bla_{VIM-4}*. Eine molekulare Typisierung mittels cgMLST zeigte, dass 3 Isolate am ehesten Teil eines Ausbruchs waren während die anderen vierzehn Isolate epidemiologisch nicht verwandt waren. Anschließend wurde die genetische Plattform der MBLs durch S1 Pulsed Field-Gelelektrophorese und Southern Blot identifiziert. Zehn Isolate besaßen MBL-kodierende Plasmide (alle Isolate, in denen *bla_{GIM-1}* und *bla_{VIM-2}* und drei der Isolate, in denen *bla_{NDM-1}* gefunden wurde), wohingegen bei sieben Isolaten die MBLs chromosomal kodiert wurden (alle Isolate, in denen *bla_{VIM-4}* und die restlichen Isolate, in denen *bla_{NDM-1}* gefunden wurden). Das Mapping der Plasmide wurde mittels Unicycler durchgeführt, indem aus den short read-Sequenzen des MiSeq und den long read-Sequenzen des MinION Hybrid-Assemblies erzeugt wurden. Die Konjugation der Plasmide in *E. coli* und *A. baumannii* wurde mittels „broth mating“ überprüft. Die Konjugation in *E. coli* war nicht erfolgreich, während alle Plasmide, die *bla_{NDM-1}* enthielten und vier der fünf Plasmide, die *bla_{GIM-1}* enthielten, in *A. baumannii* konjugativ waren. Obwohl die Isolate klinisch nicht verwandt waren, zeigten die Plasmide eine hohe Ähnlichkeit. Das genetische Grundgerüst der Plasmide zeigte eine Übereinstimmung von >99%. Signifikante Unterschiede imponierten

lediglich in der MBL-kodierenden Region. Die *bla*_{NDM-1}-enthaltenden Plasmide zeigten hohe Ähnlichkeit zu anderen Plasmiden, die weltweit in *Acinetobacter* beschrieben worden sind, insbesondere in Asien und Nord- und Südamerika, wohingegen die *bla*_{VIM-2}- und *bla*_{GIM-1}-kodierenden Plasmide bislang noch nicht beschrieben wurden. *bla*_{VIM-2}- und *bla*_{GIM-1} waren in einem Cluster von Resistenzgenen angeordnet, welches von einer Integrase (*Int11-like*) flankiert wurde und Ähnlichkeit zu Transposon *TnAs3* besaß. Daher stellten wir die Hypothese auf, dass die *bla*_{VIM-2}- und *bla*_{GIM-1}-kodierenden Plasmide Varianten des ursprünglichen, *bla*_{NDM-1}-kodierenden Plasmids sind, die eine Integrase erworben haben – möglicherweise mittels eines Transposons – welche anschließend verantwortlich für die Integration von Resistenzgen-tragenden Genkassetten in das Plasmid war. Während das *bla*_{NDM-1}-kodierende Plasmid bereits weltweit in Isolaten identifiziert werden konnte, ist diese Studie die erste, die Varianten dieses Plasmids beschreibt, die eine andere MBL abgesehen von *bla*_{NDM-1} enthalten. Dies könnte darauf hinweisen, dass diese neuen Plasmidvarianten ihren Ursprung in Deutschland haben. Weitere Forschung bezüglich MBL-kodierender *A. pittii* in anderen europäischen Ländern könnte ein genaueres Bild der aktuellen Verteilung der Resistenzmuster in *Acinetobacter* in Europa zeichnen und die Prävalenz von Plasmidvarianten des ursprünglichen *bla*_{NDM-1}-kodierenden Plasmids klären.

Es ist wichtig zudem zu beachten, dass bei zukünftiger, komparativer Plasmidanalyse aufgrund der außergewöhnlichen Genomplastizität von *Acinetobacter* und der Kompatibilität von mobilen genetischen Elementen zwischen verschiedenen *Acinetobacter* Spezies nicht nur eine einzelne Spezies mit einbezogen werden sollte. Des Weiteren sollte die Plasmidanalyse, insbesondere die Analyse des Resistoms, unabhängig von der Analyse des gesamten bakteriellen Genoms durchgeführt werden, da bislang verwandte Plasmide in nicht verwandten Wirtsbakterien gefunden werden.

3. Introduction

3.1. The genus *Acinetobacter*

The genus *Acinetobacter* belongs to the *Moraxellaceae* family within the order *Gammaproteobacteria* and comprises a heterogeneous group of Gram-negative, strictly aerobic, glucose non-fermenting, oxidase-negative, catalase-positive bacteria.¹ The cells measure about 1.5 µm in length and are of coccoid to coccobacillary shape (Figure 1).² *Acinetobacter* species occur ubiquitously in the environment and have been commonly isolated from soil and water samples.³⁻⁶ Furthermore, isolates have been recovered from alimentary plants, such as peppers⁷, lettuce⁸ and tomatoes⁹, in livestock-associated settings such as animal housing¹⁰, swine manure¹¹ or isolated directly from pigs and cattle¹² as well as in wild birds¹³. MDR isolates are, however, rarely found in the environment. In two studies *Acinetobacter* isolates were detected as commensal inhabitants of the human skin in 42.5% and 44% of non-hospitalised individuals respectively. One of the two studies found that up to 75% of hospitalized patients were colonized with *Acinetobacter* with both percentage of colonized patients and the number of colonised locations increasing with length of hospitalization. *A. Iwoffii* was found most frequently, followed by *A. johnsonii*, *A. radioresistens* and *A. pittii*.^{14,15} Furthermore *Acinetobacter* has been found in human faeces in non-hospitalised individuals with *A. johnsonii* as the most prevalent species, followed by *A. guillouiae*.¹⁶ Nosocomial infections with *Acinetobacter* are most commonly caused by *A. baumannii* as well as *A. pittii* and *A. nosocomialis*.^{17,18} Other species are rarely found as etiologic agents in nosocomial infections and are mainly limited to catheter-associated infections due to their presence on human skin.^{2,19}

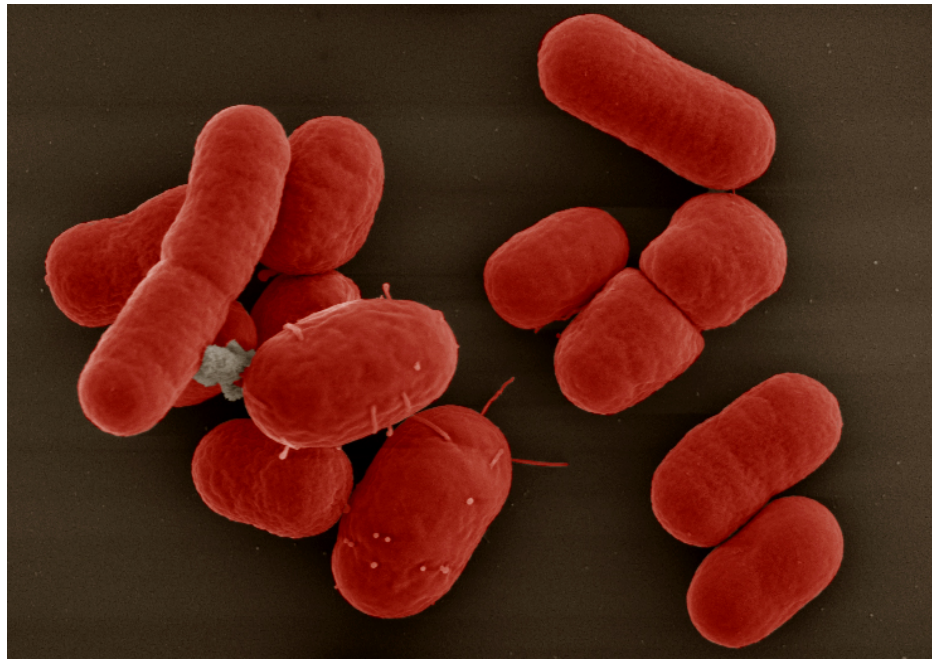


Figure 1. *Acinetobacter baumannii* in electron microscopy

The picture was taken from the website of the Robert Koch institute, Berlin, Germany.²⁰

3.1.1. History

The taxonomic history of the genus *Acinetobacter* has been subject to debate and change throughout the years. The first record of an *Acinetobacter* isolate dates back to 1911, when Dutch microbiologist Beijerinck isolated a bacterial organism from soil. He went on to name it *Micrococcus calco-aceticus*.²¹ The current name, *Acinetobacter* - stemming from the Greek word ακινετος [akinetos] for “nonmotile” - was proposed in 1954 by Brisou and Prévot in an attempt to distinguish nonmotile from motile bacteria in the *Achromobacter* genus.²² It was subsequently redefined by Baumann et al. in 1968 to designate oxidase-negative specimen from the *Moraxella* group, who additionally stated that a further subclassification based on phenotypic criteria was not possible.²³ In 1986, by using DNA-DNA hybridization Bouvet and Grimont were able to identify 12 different genospecies amongst representatives of the *Acinetobacter* genus and proposed three new species names, *A. baumannii* for genospecies 2, *A. haemolyticus* for genospecies 4 and *A. junii* for genospecies 5 in addition to the two already validly termed species: *A. calcoaceticus* (genospecies 1) and *A. Iwoffii* (genospecies 8).²⁴ Further research by Bouvet and Jeanjean as well as Tjernberg and Ursing in 1989 resulted in the description of additional species (labelled BJ and TU respectively), which in some cases later on turned out to be synonyms of each other.^{25,26} As of now, 87 distinct, validly named *Acinetobacter* species have been identified.^{27,28}

3.1.2. The *A. calcoaceticus* – *A. baumannii* complex (ACB complex)

Because of their close genetic relatedness *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomospecies 3 and genomospecies 13TU (in 2011 named *A. pittii* and *A. nosocomialis*, respectively²⁹) are difficult to distinguish from each other by routine diagnostic phenotypic testing.¹⁸ They were hence grouped together in the so-called “*A. calcoaceticus* – *A. baumannii* complex”.^{30,31} The taxonomic study by Tjernberg and Ursing in 1989 suggested the idea, that the ACB complex represents a subgroup within the *Acinetobacter* genus as these species were more closely related to each other than to other *Acinetobacter* spp.²⁶ Further investigation into the matter by Gerner-Smidt and Tjernberg found strains representative of two new DNA groups within the ACB complex, one of them close to genomospecies 13TU and the other close to DNA groups 1 and 3.³² In 2015 the group, which was formerly termed “close to 13TU”, was identified as a distinct species and subsequently named *A. seifertii*.³³ In 2016 two publications introduced *Acinetobacter lactucae* and *Acinetobacter dijkshoorniae* as novel species from the ACB complex and both were closely related to *A. pittii*.^{8,34} In 2018, however, the isolates from both species were found to be in fact conspecific and *A. dijkshoorniae* was defined as a later synonym of *A. lactucae*.³⁵ Although the grouping of species in the ACB complex makes sense from a taxonomic viewpoint, it comprises not only the most clinically significant *Acinetobacter* spp., but also an environmental species, *A. calcoaceticus*, which is misleading from a clinical point of view. Hence, the term “*A. baumannii* group” was proposed to denote the clinically relevant species of the ACB complex, as they are similar in epidemiological and clinical determinants.¹⁸

3.1.3. *Acinetobacter* spp. as clinical pathogens

Members of the *A. baumannii* group, and *A. baumannii* in particular, have proven to be important, clinically relevant pathogens. In addition, *Acinetobacter* have shown a propensity to persist on inanimate surfaces in hospital settings, thus posing to be an ongoing originator of nosocomial transmission.^{36,37} *A. baumannii* has shown remarkable desiccation tolerance. In a study by Jawad et al. survival times of *A. baumannii* on dry surfaces were shown to range between 21 and 33 days with a mean survival time of around 27 days.³⁸ A study examining *A. radioresistens* has demonstrated an even more extensive ability to survive on dry surfaces.³⁹ Furthermore, all members of the *A. baumannii* group have shown the capability to form biofilms additionally protecting them from desiccation and further enhancing their capacity of survival.⁴⁰⁻⁴³ A study of desiccation in *A. pittii* showed a significant desiccation tolerance and ability to form biofilms as well.⁴⁴

The most common nosocomial infection caused by members of the *A. baumannii* group and particularly *A. baumannii*, is hospital-acquired pneumonia, especially in ventilated patients. This has become particularly relevant in view of the current COVID-19 pandemic, which saw high numbers of patients being subject to artificial ventilation. But as of late also infections of the nervous system, soft tissue and bones have been described as well as urinary tract and blood stream infections.¹⁸ Infections target mostly chronically ill and immunocompromised patients in an ICU setting, and these infections can be the source of healthcare-associated outbreaks. Coinfection of SARS-CoV-2 patients with *A. baumannii* has been reported multiple times in Europe, Asia and South America with high prevalences of carbapenem-resistant organisms.⁴⁵ Infections have also been observed in trauma patients, often in succession of natural catastrophes or combat activities.⁴⁶ Community-acquired infections with *Acinetobacter* spp. are rare, but have been observed in subtropical and tropical regions and afflict patients with co-morbidities such as chronic lung or renal disease, alcoholism, diabetes, malignant tumours and in immunocompromised patients.⁴⁷⁻⁴⁹ The impact on mortality mediated by *Acinetobacter* infections is still the subject of debate. Studies researching this association have difficulties in coming to general conclusions, as the methodology in study design has been quite heterogenous and patients often suffer from co-morbidities, which renders clear associations difficult.^{18,50,51}

A. baumannii still appears to be the most prevalent species of the genus, however, prevalence of *A. pittii* has increased in recent years.^{52,53} Although case reports of *A. pittii* infections are still scarce in comparison to *A. baumannii*, bloodstream infections with *A. pittii* were frequently reported.⁵⁴⁻⁵⁸ A few studies even depict a higher prevalence of *A. pittii* over *A. baumannii* among bloodculture isolates.^{56,57} Reports of respiratory infections caused by *A. pittii*, describe cases in both immunocompetent as well as immunocompromised patients.⁵⁹⁻⁶³ Consistent with the reports of *A. pittii* as an inhabitant of human skin, nosocomial infections caused by medical procedures or extraneous material were also reported.^{64,65} A neurological infection caused by *A. pittii* has so far been described only once.⁶⁶

3.2. Antibiotic resistance in *Acinetobacter*

Members of the genus *Acinetobacter* appear to have the potential to develop resistance to antimicrobials more rapidly than other clinically relevant bacteria, which might be attributed to its evolutionary exposure to antibiotic compounds produced by other organisms encountered in their natural soil habitat.⁶⁷ The most prominent representative of this genus, *Acinetobacter baumannii*, has become a serious threat to antibiotic efficiency, because of its propensity to acquire resistance to a broad variety of antimicrobials and its ability to up-regulate intrinsic antibiotic resistance mechanisms such as efflux.⁶⁸⁻⁷⁰ Most studies on *Acinetobacter* have focused on this species and significantly fewer on other *Acinetobacter* spp. such as *A. pittii*, because of their lower prevalence and lower resistance rates.⁷¹ Although antimicrobial resistance in *Acinetobacter* non-*baumannii* species within the *A. baumannii* group appears to be less common,⁷² emergence of carbapenem resistance in *A. pittii* has been noted worldwide.⁷³⁻⁷⁵ Even multi-drug resistant (MDR), extensively drug-resistant (XDR) or pan-drug resistant (PDR) *A. pittii* have been reported with increasing frequency, mostly in Asia.^{61,71,76-81} These studies indicate, that other *Acinetobacter* spp. from the *A. baumannii* group, in particular *A. pittii*, are clinically relevant and of increasing concern.

3.2.1. Antimicrobial agents and resistance mechanisms

A broad variety of antibiotics can be used to treat infections with Gram-negative bacteria.⁸² Antibiotics can be either classified as bacteriostatic (inhibiting bacterial growth) or bactericidal (effectively killing bacteria).⁸³ Bacteriostatic antibiotics active in Gram-negative bacteria include tetracyclines (e.g. tetracycline), glycylicyclines (e.g. tigecycline), macrolides (e.g. azithromycin), sulphonamides (e.g. sulfamethoxazole) and others compounds like chloramphenicol and trimethoprim. Bactericidal antibiotics used in Gram-negative infections include β -lactams comprising the groups of penicillins (e.g., amoxicillin, piperacillin), cephalosporins (e.g., cefazolin, ceftriaxone), carbapenems (e.g. imipenem, meropenem) and monobactams (aztreonam) as well as aminoglycosides (e.g., amikacin, gentamicin, tobramycin), fluoroquinolones (e.g., ciprofloxacin), rifamycins (e.g., rifampicin), polymyxins (e.g., colistin) and further compounds such as fosfomycin.⁸⁴ Resistance mechanisms towards these agents generally fall into one of three categories: 1. inactivation of antimicrobials through enzymes, 2. reducing access to bacterial targets (through efflux pumps or by decreasing drug uptake) 3. mutations of target binding sites or cellular functions.⁸⁵ Furthermore, resistance to antibiotic agents can be classified as either intrinsic resistance, designating inherent resistance to certain antimicrobial classes, or extrinsic resistance, indicating resistance mechanisms that have been acquired by the bacteria. The resistance armamentarium in *Acinetobacter* spp. is ample and rivals those of other nonfermentative Gram-negative bacteria (Table 1).¹⁸

Table 1. Antibiotic groups and respective resistance mechanisms in *Acinetobacter* (table amended from Gerson et al. with permission)⁸⁶

Antibiotic group	Resistance mechanism	Function
β-lactams	β-lactamases (e.g. NDM, ADC, OXA) ^{17,18,87}	modification/inactivation of antibiotic agent
	altered outer membrane proteins (OMPs) (e.g. CarO) ⁸⁸	hindering access to target
	altered penicillin-binding proteins (PBPs) ⁸⁹	mutation of target binding site
Aminoglycosides	aminoglycoside-modifying enzymes ^{90,91}	modification/inactivation of antibiotic agent
	ribosomal methylation (RmtA, RmtB, ArmA) ⁹²	hindering access to target
Quinolones	mutation in <i>gyrA</i> and <i>parC</i> ^{93,94}	mutation of target binding site
	proteins of the pentapeptide family (<i>qnr</i> -genes) ⁹⁵	target site protection
	acetylation by <i>aac(6′)-Ib-cr</i> ⁹⁵	modification/inactivation of antibiotic agent
	enhanced efflux (efflux pumps QepAB & OqxAB) ⁹⁵	reduction of intracellular antibiotic concentration
Tetracyclines & glycyclcyclines	Specific efflux (e.g. Tet(A), Tet(B), Tet(H)) ⁹⁶	reduction of intracellular antibiotic concentration
	ribosomal protection (e.g. Tet(M)) ⁹⁷	hindering access to target
	flavin-dependent monooxygenase (e.g. Tet(X)) ⁹⁸	modification/inactivation of antibiotic agent
Sulphonamides	Dihydropteroate synthases (<i>sul1</i> , <i>sul2</i>) ⁹⁹⁻¹⁰¹	target mutation
Polymyxins	Loss or mutation of lipid A in lipopolysaccharide (e.g. through mutation in <i>lpxA</i> , <i>lpxC</i> and <i>lpxD</i> ¹⁰²)	loss/mutation of target binding site
	Mutations in PmrAB-regulatory system ¹⁰³	modification of target binding site

Macrolides	Phosphotransferases (e.g. <i>mph(E)</i>) ¹⁰⁴	modification/inactivation of antibiotic agent
	Specific efflux (e.g. <i>msr(E)</i>) ¹⁰⁴	reduction of intracellular antibiotic concentration
Rifamycins	Mutations in <i>rpoB</i> ¹⁰⁵	modification of target binding site
	ADP-ribosyltransferase Arr-2 ^{106,107}	modification/inactivation of antibiotic agent
Chloramphenicol	Specific efflux (<i>CraA</i>) ¹⁰⁸	reduction of intracellular antibiotic concentration
	Chloramphenicol acetyltransferases (<i>cat</i>) ⁹⁹	modification/inactivation of antibiotic agent
Fosfomycin	MurA mutation ¹⁰⁹	hindering access to target
	genes encoding for inactivating enzymes (<i>fosX</i>) ¹⁰⁹	modification/inactivation of antibiotic agent
	putative salvage pathway in peptidoglycan recycling ¹¹⁰	cellular function
	Specific efflux (<i>AbaF</i>) ¹¹¹	reduction of intracellular antibiotic concentration
Trimethoprim	Mutations in dihydrofolate reductase (<i>dfr</i>) ¹¹²	modification of target binding site
Various	Overexpression of multi-drug efflux (e.g. AdeABC, AdeFGH, AdeM) ¹¹³	reduction of intracellular antibiotic concentration

3.2.2. Betalactam antibiotics

After Alexander Fleming's discovery of penicillin in the 1920s¹¹⁴, a whole armada of penicillin derivatives and other antibiotic classes in the β -lactam group have been developed. All of these antibiotics are characterised by the eponymous four-membered heterocyclic ring and can be subdivided into penicillins (e.g. amoxicillin, piperacillin), cephalosporins (e.g. ceftriaxone, cefazoline), carbapenems (e.g. imipenem, meropenem), monobactams (e.g. aztreonam) and β -lactamase-inhibitors (e.g. sulbactam, clavulanic acid), although some newer agents in the last group do not possess the β -lactam-ring (e.g. avibactam).¹¹⁵ β -lactam antibiotics are bactericidal and function by impeding cell wall formation in bacteria by binding to so-called penicillin-binding proteins (PBP).¹¹⁶ The first β -lactam used in a clinical setting was penicillin G, utilized mostly in order to treat streptococcal infections.¹¹⁷ However, bacterial β -lactamases in staphylococci challenged penicillin effectiveness, which led to the development of

penicillinase-stable penicillin-derivatives as well as other β -lactam-groups.¹¹⁸ Nowadays, β -lactam antibiotics are the most commonly employed antibiotic class and are used in a broad variety of settings. In the United States they accounted for 65% of all injectable antibiotics between 2004 and 2014.¹¹⁸ Hence, the increasing resistance to these agents is of particular concern. β -lactamases, which are the most common source of β -lactam-resistance in *Acinetobacter*, can be grouped using the Ambler classification dividing β -lactamases into four classes based upon their amino acid sequence (Table 2).^{119,120} The Ambler classification was introduced in 1980 by Richard Penry Ambler, an English molecular biologist, who initially differentiated β -lactamases of class A, utilising serine at the active site and class B metallo- β -lactamases, utilising divalent zinc ions for hydrolysis.^{121,122} Later on, two new classes of serine β -lactamases (class C and D) were described, which bore little similarity to those of class A, however, and were thus identified as distinct classes.¹²³⁻¹²⁵ Similarly to *A. baumannii*, *A. pittii* naturally possess two chromosomally encoded β -lactamases: an Ambler class C cephalosporinase (*bla_{ADC}*)⁸⁵ and an Ambler class D oxacillinase (*bla_{OXA-213-like}*)⁷⁵. Generally *Acinetobacter* spp. are intrinsically resistant to penicillins and first and second generation cephalosporins.¹²⁶ Hence, because of their broad spectrum as well as favourable safety and tolerability profiles, carbapenems are the most common treatment for infections with *Acinetobacter*, which often turn out to be multi-drug resistant.¹²⁷ Due to acquired resistance mechanisms, however, carbapenem-resistance in *Acinetobacter* has become increasingly frequent.¹²⁸⁻¹³¹ The most frequently found β -lactamases in *Acinetobacter* are Ambler class D β -lactamases, but as of late, also Ambler class B metallo- β -lactamases (MBLs) have been found to be mediators of carbapenem resistance.¹²⁶

Table 2. Ambler classification of β -lactamase enzymes.

Ambler class and subclass		Mechanism of action	Common enzymes
A		serine β -lactamase	CTX-M, GES, KPC, SHV, TEM
B	B1	metallo β -lactamase	BclI, DIM, GIM, IMP, KHM, NDM, SIM, SPM, TMB, VIM
	B2		CphA, ImiS, SFH
	B3		AIM, BJP, CAR, CAU, FEZ, GOB, L1, POM, SMB, THIN
C		serine β -lactamase	ACT, ADC, CMY, DHA
D		serine β -lactamase	OXA

3.2.3. Metallo- β -lactamases

MBLs are class B β -lactamases that are able to hydrolyse almost all clinically available β -lactam antibiotics including carbapenems, with the exception of monobactams.¹³² The group is heterogenous and different MBL groups show very low sequence identities, however, all are consistent in sharing a zinc (Zn) binding active site.¹³³ The Ambler class B has been further subdivided into subclasses B1 - B3 by means of amino-acid sequence and structure.¹³⁴ MBLs from groups B1 and B3 require two divalent Zn ions bound to their active site (Figure 2) and are capable of hydrolysing penicillins, cephalosporins and carbapenems.¹³³ B2 MBLs, on the other hand, only require one divalent Zn ion and are only potent towards carbapenems.¹³³ The first MBL (BcII from *Bacillus cereus*) was discovered in 1965 by Sabath and Abraham.¹³⁵ Initially, however, MBLs were mostly found in non-pathogenic organisms and were only chromosomally encoded. They were hence considered to be of low clinical relevance.¹³⁶ This drastically changed with the emergence of IMP- and VIM-type MBLs (imipenemase and Verona imipenemase) in Gram-negative pathogens in the 1990s.^{137,138} Ever since, genes coding for MBLs have been increasingly associated with mobile genetic elements, facilitating their dissemination. In consequence, MBLs have become significant mediators of clinical carbapenem resistance.¹³⁹ These clinically relevant MBLs, such as DIM, FIM, GIM, IMP, KHM, NDM, SIM, SPM, TMB and VIM, mostly stem from Ambler subclass B1 and share about 23% amino acid identity.¹³³ AIM is so far the only known MBL from another subclass, subclass B3, that has been described in clinically relevant isolates.¹⁴⁰ The most commonly found MBLs in *Acinetobacter* are the IMP-, NDM- and VIM-type groups, which have been reported in *Acinetobacter* spp. worldwide.¹⁴⁰

The first identified transferable MBL was the imipenemase-type MBL (IMP), which was found in 1988 in a *Pseudomonas aeruginosa* isolate in Japan.¹⁴¹ IMP-family MBLs have since been found in a multitude of Gram-negative species in countries all over the world.¹⁴² IMP variants share between 80 and 99.6% sequence identity.¹³³ The first occurrences of IMP in Europe were described in *A. baumannii* isolates, first in Italy (*bla*_{IMP-2}) and later in Portugal (*bla*_{IMP-5}).¹⁴³⁻¹⁴⁵ In *A. pittii*, IMP-carrying isolates have been described in multiple countries all over the world, with most reports coming from Asia.¹⁴⁶⁻¹⁴⁸

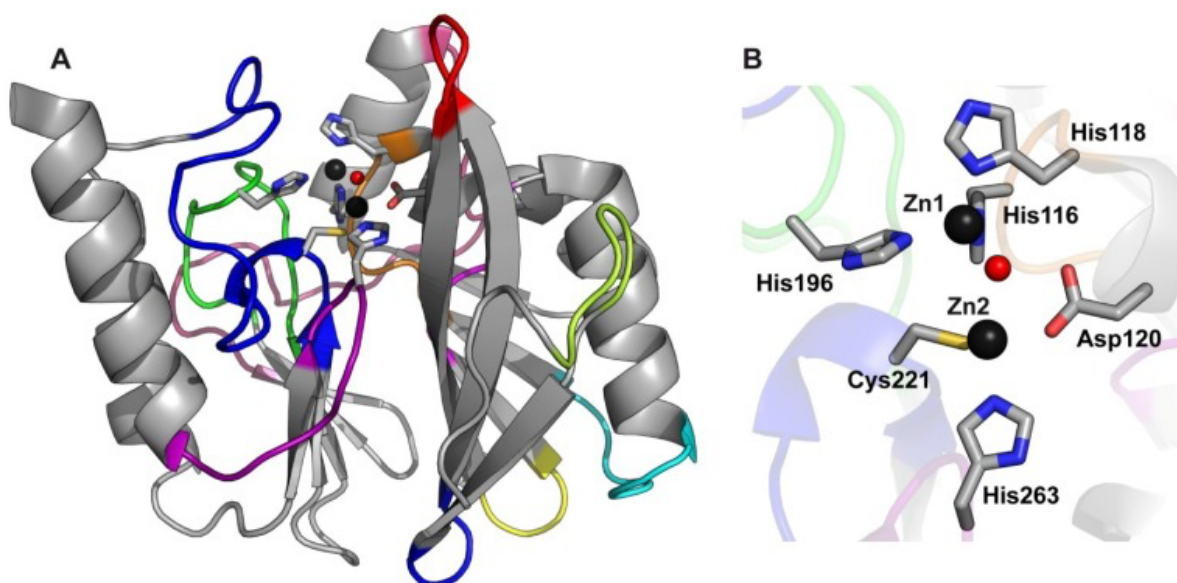


Figure 2. Fold structure and active site of VIM-2

(A) Crystallographic structure

(B) Active site with two Zn(II) ions (black) bound by ligand amino acids and H₂O (red)

(taken from Meini et al.¹³³)

The first Verona integron-encoded metallo-β-lactamase (VIM-1) was discovered in 1997 in Verona in a *Pseudomonas aeruginosa* isolate and exhibited the highest resemblance to BclI.¹³⁸ Soon after, the variant VIM-2 was reported from an isolate found in 1996 in France, which showed 90% amino acid identity to its predecessor.¹⁴⁹ The variant VIM-4 is even more similar to VIM-1 and differs only in one amino acid.¹⁵⁰ Since then 80 VIM-variants have been identified. Sequence identities in between VIM-type MBLs range from 72.9% to 99.6% with VIM-7 being the most divergent variant.¹³³ Although the identification of VIM-type MBLs has increased in the *Acinetobacter* genus, reports on VIM-carrying *A. pittii* are scarce.^{79,151,152}

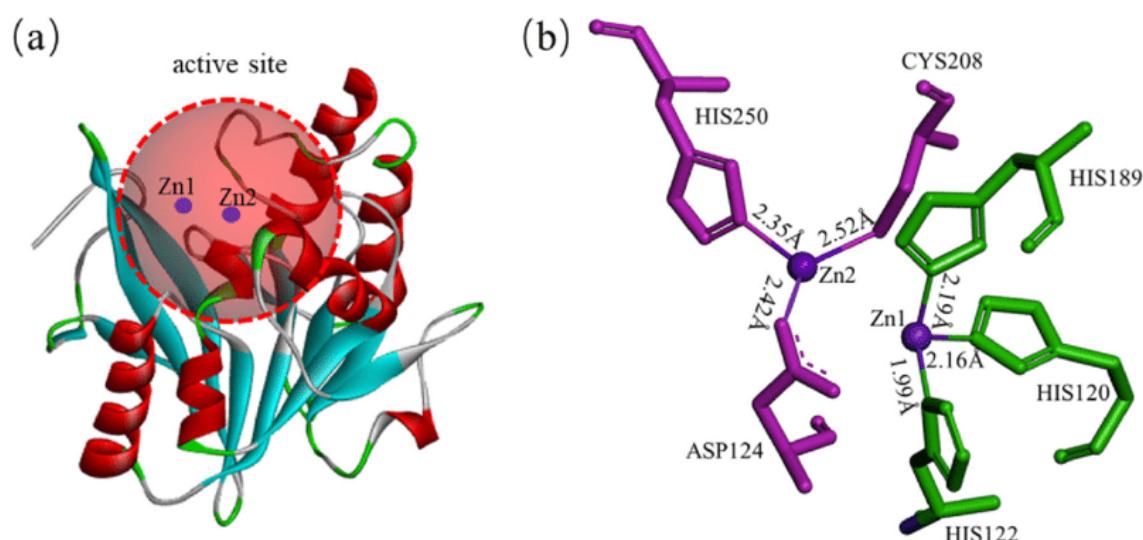


Figure 3. Fold structure and active site of NDM-1

(A) Protein folding of NDM-1; active site (red)

(B) Active site of NDM-1 with two Zn(II) ions (black) bound by ligand amino acid (taken from Li et al.¹⁵³)

The occurrence of the New Delhi metallo-β-lactamase (NDM) was first documented in a *Klebsiella pneumonia* isolate in 2008.¹⁵⁴ Since then, there have been reports about its occurrence in many Gram-negative species from more than 40 countries, some of which even predate 2008. Diverse plasmids harbouring *bla*_{NDM} have been described, showing the ability for transmission to other bacterial species and genera.¹⁵⁵ Although the spread of NDM was initially thought to be epidemiologically associated with the Indian subcontinent,¹⁵⁶ *bla*_{NDM-1} has also been identified in an *A. baumannii* isolate from a German patient who was repatriated from Serbia in 2007, suggesting that bacteria expressing NDM-1 might have been more widely dispersed than originally believed.^{157,158} NDM-variants are less diverse than those of the other MBLs, often differing in only one or two amino acids.¹³³ In *A. baumannii*, NDM-1 is found widespread in isolates worldwide.^{159,160} Similarly, in *A. pittii* *bla*_{NDM-1} is becoming more and more frequent, and has been found in isolates in Asia, the Middle East, as well as Europe and South America as of now.¹⁶¹⁻¹⁶⁵

Another MBL, the first German imipenemase (GIM-1) was discovered in five *P. aeruginosa* isolates from Germany in 2002.¹⁶⁶ Its amino acid sequence showed most similarity to IMP-type variants 6, 1 and 4. Only one variant of GIM-1, GIM-2, has been found as of now.¹⁶⁷ In the *Acinetobacter* genus GIM-type enzymes have so far been rarely documented^{168,169} and there has up to this point been only one report of *A. pittii* isolates harbouring *bla*_{GIM-1}, of which 5 isolates are also included in this work.¹⁷⁰

3.3. Horizontal gene transfer

Horizontal gene transfer (HGT) is an umbrella term for a variety of different mechanisms (Figure 3), that enable bacterial cells to transfer single- or double-stranded DNA from one cell to another. It has been a staple in bacterial genome plasticity, thus significantly contributing to bacterial evolution.¹⁷¹ Conjugation was the first described example of HGT and was found initially in an *Escherichia coli* sample in 1947.^{172,173} Since then, detection of HGT has steadily improved and has unmasked the astonishing prevalence as well as the epidemiological relevance of these mechanisms.¹⁷⁴ In bacteria, the horizontal transfer of antibiotic resistance genes (ARGs) has become a major vehicle for adaptation to new antimicrobial agents.¹⁷⁵ It has been important in the propagation of antimicrobial resistance (AMR) and providing a selective advantage to bacterial pathogens.¹⁷⁶ The three most recognised methods of HGT are transformation, conjugation and transduction, but in more recent studies, further mechanisms have been detected, such as HGT via gene transfer agents (GTAs), outer membrane vesicles (OMVs) or nanotubes.¹⁷¹

Transformation (Figure 3a) describes the uptake and integration of naked DNA fragments from the extracellular environment. It has been shown in particular, that antibiotic stress – besides selecting resistant strains in bacterial colonies – also induces bacteria to garner ARGs through transformation, supporting AMR development.¹⁷⁷ Domingues et al. have demonstrated that some mobile genetic elements (MGEs) such as integrons, gene cassettes and transposons can thus be transmitted between species with little regard to genetic compatibility. Therefore, transformation represents an effective mechanism for broad horizontal gene acquisition.¹⁷⁸ As there is a great plethora of extracellular DNA available to environmental specimens, this DNA can in this context be considered as a reservoir to bacteria competent for transformation.¹⁷⁹

Conjugation (Figures 3b & 3c), the most studied HGT, on the other hand, requires direct physical contact between two bacterial cells in which one cell acts as the donor and the other one as the recipient. The required connection is arranged through proteins of a type 4 secretion system (T4SS), called the mating pair formation (MPF) complex. By means of this thus established canal, genetic material is transferred between isolates, providing better protection to the genetic material than transduction or transformation. Additionally to genes for the MPF complex, a set of mobility genes as well as a relaxase are necessary for HGT through conjugation.¹⁸⁰ T4SS are a structurally and functionally diverse family of membrane-spanning transport systems, that can be grouped into three subfamilies depending on their function.¹⁸¹ First off are the already described T4SS posing as conjugation systems, enabling DNA transfer between two bacterial cells. Besides, there are ‘DNA uptake and release’ systems, that mediate DNA transfer into or out of a single bacterial cell and lastly, ‘effect translocator’ systems, that deliver DNA or proteins to eukaryotic target cells by means of an infection

process.^{182,183} The first description of the structure of a T4SS derives from studies on *Agrobacterium tumefaciens*.¹⁸⁴ This prototype system comprises 12 core components (termed VirB1 – VirB11 and VirD4).¹⁸⁵ This set of 12 proteins is also known as the “minimized T4SS” in Gram-negative bacteria, whereas a “minimized system” in Gram-positive bacteria on the other hand consists of only six components.^{183,186} Some bacteria possess “expanded T4SS”, which are composed of not only the core components of the minimized system, but additional system-specific others.^{181,187} There are two key vectors of intercellular mobility that are available to conjugation: plasmids and integrative conjugative elements (ICEs). Plasmids are extra-chromosomal, circular units of DNA, that can range from 1.3 up to 400 kbp in size in the *Acinetobacter* genus.⁸⁹ They are capable of replication by binding of a Rep protein to the origin of replication (*oriV*). The Rep protein is encoded by a *rep* gene, which is commonly located on the plasmid itself.^{188,189} For other proteins needed in replication (helicase, primase, polymerase) plasmids usually take advantage of the corresponding genes located on the chromosome.¹⁸⁸ In order to distinguish different plasmid groups in resistance plasmids from *A. baumannii* isolates, a PCR-based replicon typing method (AB-PBRT) was designed, which creates groups on similarities in the plasmids’ Rep proteins.¹⁹⁰ Based on this typing method, 19 distinct groups (GR1-GR19) were initially established and further groups (GR20-GR24) were described later.^{89,191-193} There are, however, a few known plasmids in *Acinetobacter* spp., like pRAY- and pNDM-BJ01-derivatives, that do not harbour a Rep protein and can therefore not be classified by the AB-PBRT method.^{188,194} Plasmids, that encode their own set of MPF genes and are as a result self-transmissible, are called conjugative plasmids. Plasmids are termed mobilisable if they are able to utilise MPF genes from another element in the cell but are not transmissible on their own. If neither is the case, the plasmids are deemed non-mobilisable and are only available to transmission by other HGT mechanisms.^{180,195} ICEs on the other hand are typically found on the chromosome and like conjugative plasmids carry the genes necessary for conjugation, as well as genes for integration and excision. ICEs can be excised from chromosomal DNA and subsequently be conjugated to another cell and then reintegrate in the recipient’s chromosome (Figure 4d).¹⁹⁶ As multiple ARGs are often associated with the same conjugative elements, multi-drug resistance disseminated through conjugation is common. The fact that similar conjugative elements have been found in unrelated bacteria all over the world indicates that this mechanism contributes significantly to the worldwide dissemination of antimicrobial resistance.¹⁷⁷

Transduction describes the transmission of genes by means of bacteriophages and does not need direct cell-to-cell contact. As ARGs have been increasingly detected in bacteriophages in environmental as well as in clinical samples, transduction appears to be more common than initially believed.¹⁹⁷⁻¹⁹⁹ ARG carrying environmental organisms can be considered reservoirs for bacteria and bacteriophage appear to have a particularly wide host range, making them an exceptionally competent tool in ARG dissemination.^{177,200} Similar to findings in transformation, there is also evidence showing an augmentation of transduction through antibiotic treatment.²⁰¹

HGT via GTAs, whose mechanism is similar to transduction, has been demonstrated for the first time in *Rhodobacter capsulatus* in 2000.²⁰² GTAs are structures resembling bacteriophages that are produced by their host cell. They carry random genetic material of the cell's genome and are released by cell lysis. The amount of genetic material packaged by a GTA is however too small to encode for all proteins necessary for GTA production, meaning that GTAs themselves are not able to transport all genes to another cell, that would be necessary for GTA-forming.²⁰³ The process of GTA production is solely regulated by the cell itself.²⁰⁴⁻²⁰⁶ GTA transfer of ARGs has been demonstrated, however, the impact on human health is yet to be determined.¹⁷⁷ GTAs are mainly found in *Alphaproteobacteria* and have not yet been described in *Acinetobacter*.²⁰⁷

OMVs, another recently discovered mechanism of HGT, are vesicles that are produced by growing cells and can transfer proteins, lipopolysaccharides, phospholipids, RNA as well as DNA through partition of the vesicle from the host cell and reintegration into the recipient cell.²⁰⁸ HGT of ARGs via OMVs has been reported in *A. baumannii* where dissemination of a class D oxacillinase-encoding gene was described.²⁰⁹ Antibiotic stress also appears to induce the formation and increase the DNA content of OMVs.²¹⁰ Additionally, Yaron et al. have found entire plasmids inside OMVs suggesting them as one potential mechanism responsible for the dissemination of non-conjugative plasmids.²⁰⁸

Nanotubes, on the other hand, deploy an operating mode similar to that of conjugative pili, as DNA can be transferred via a canal-like structure directly from one bacterium to another via cell-cell contact.²¹¹ Nanotubes can even transfer nonconjugative plasmids between cells and can also establish connection in-between bacteria of different species.^{211,212} Contrary to conjugation, however, this mechanism is not associated with formation of a T4SS.²¹³ Apart from this function nanotubes can serve for cell-cell communication and can also transport proteins in between communicating cells.²¹⁴ There has only been one study on nanotube formation in *Acinetobacter*, where development of these structures could not be demonstrated. So, it remains unclear, if *Acinetobacter* spp. possess the ability to use nanotubes for HGT.

Up until today conjugation appears to be the most common mechanism of HGT in *Acinetobacter* especially when talking about HGT of ARGs, which does not come as a big

surprise as it is also the most well researched. Nonetheless, HGT has been a subject of significant recent interest and plenty of new insight into both known, as well as unknown mechanisms, has been gathered in the last couple of years. Hence, new research might further elucidate, if and to what extent other HGT mechanisms play a role in bacteria from the genus *Acinetobacter*.

Figure 4. Schematic display of main mechanisms of HGT in bacteria

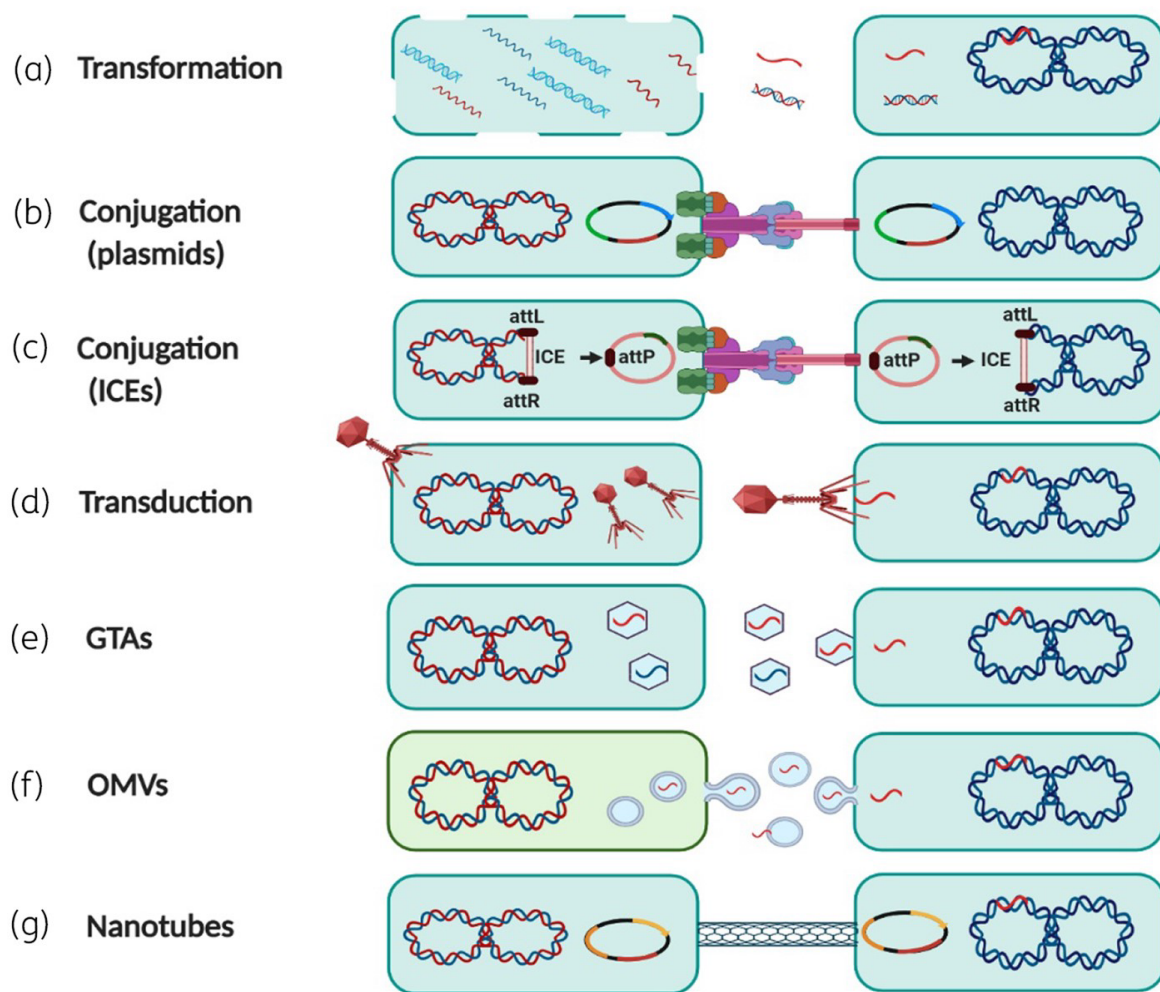


Figure 4. Main mechanisms of HGT in bacteria

(a) Transformation: bacterial uptake of extracellular (naked) DNA

(b) & (c) Conjugation: uptake of genetic material either in form of plasmids or integrative ICEs through a mating-pore pilus mediated by the T4SS

(d) Transduction: transfer of DNA from one bacterium to another by bacteriophages

(e) GTAs: phage-derived sequences operate as vectors to deliver genetic material from one bacterium to another

(f) OMVs: capsules produced from the outer membrane deliver genetic material between bacteria

(g) Nanotubes: transfer of non-conjugative plasmids through nanotubes

(taken from Liu et al.¹⁷¹)

3.3.1. Mobile genetic elements and intracellular mobility

In addition to understanding the different mechanisms of HGT it is also important to recognize the various types of mobile genetic elements. Plasmids and ICEs are (as previously described) the main vectors of intercellular mobility of DNA.¹⁷¹ On the other hand, insertion sequences (IS), transposons (Tn) and integrons are the main contributors to intracellular mobility. ISs and Tns are DNA segments that can exist on both chromosomal and plasmid DNA and can translocate themselves between different locations of the same DNA molecule or between

different DNA molecules in the same cell (Figures 4 a, b, e).¹⁸⁸ Classical ISs only carry genes for one or sometimes two transposases that are required for their transposition and do not encode any other genes. IS-elements can measure between 0.7 to 2.5 kbp in size.²¹⁵ They are flanked by short terminal inverted repeat (IR) sequences of 3 to 14 bp length.¹⁸⁸ Depending on the type of IS, the transposition can occur through a conservative cut-and-paste mechanism, where the IS is excised and integrated in another spot or via replicative mechanisms.^{188,216} Replicative transposition can take place by two distinct modes. In ISs using the copy-and-paste mechanism a co-integrated molecule is formed out of donor and target. In this process the IS is duplicated and one copy each sticks to both donor and target after resolution through homologous recombination, i.e., the exchange of genetic information between two similar or identical genetic elements composed of nucleic acids.²¹⁶ Recently, another replicative mode has been described: the copy-out-paste-in mechanism. Here, an intermediate double-stranded replicate of the IS is formed which is then integrated into the recipient.²¹⁷ IS transposition can lead to a variety of effects on the host cell. If the insertion of an IS leads to the disruption of another gene, that gene can thereby be inactivated. Depending on the function of the disrupted gene this can both positively or negatively impact virulence, resistance or metabolic functions.²¹⁸ IS-elements can also provide a strong promoter that can lead to modified expression of adjoining genes. These promoters can either be part of the IS or arise through correct insertion of the IS in the target structure leading to formation of a hybrid promoter.^{188,218} There has also been research, showing that insertion of IS-elements upstream of an intrinsic ARG has drastically increased its expression and thus increased the cell's level of AMR, as has been demonstrated with *ISAbal* and *bla_{OXA-51}* like genes in *A. baumannii*.²¹⁸⁻²²⁰

When genes (in particular ARGs) are flanked by IS on both sides, they can – under certain circumstances - also be mobilized. This thus creates a structure called a “composite transposon” (Figure 4a).¹⁸⁸ Unit transposons on the other hand were originally thought to be significantly larger than IS-elements and in comparison to compound transposons, also bracketed by a pair of IR instead of ISs. They were thought to include a transposase as well, but in addition to IS-elements also carry passenger genes.¹⁸⁸ The initially supposed strict distinction between insertion sequences and transposons, however, has become blurry throughout the years, because some IS-elements (called transporter IS [tIS]) have been shown to include open reading frames (*orf*) between the transposase and the IRs, suggesting “passenger genes” that get transposed in line with the IS-element. Additionally, structures related to known Tns have been detected, that do not carry any passengers, rendering a clear distinction between different mobilizable elements (ME) difficult.^{215,221}

Integrations are characterized by a gene encoding for an integrase (*intI*), a recombination site (*attI*) and an outward-directed promoter. The site-specific tyrosine recombinase (=integrase) can facilitate translocation and integration of gene cassettes (Figure 4c) by recombining the

attC site of the cassette with the *attI* site of the integron and thereby garnering genes, often those encoding for antimicrobial resistance. Gene cassettes are small mobile elements (up to 1 kbp) consisting normally of one promoterless gene and the recombination site (*attC*). They exist in free circular form but are, unlike IS, not replicative. After integration into the integron, the outward-directed promoter is then able to operate the transcription of these captured genes. Multiple gene cassettes may be inserted into the same integron forming an array. Depending on the variant of *IntI*, different classes of integrons have been reported, with class I (harbouring *IntI1*) being the most relevant in clinical isolates.^{188,222-224}

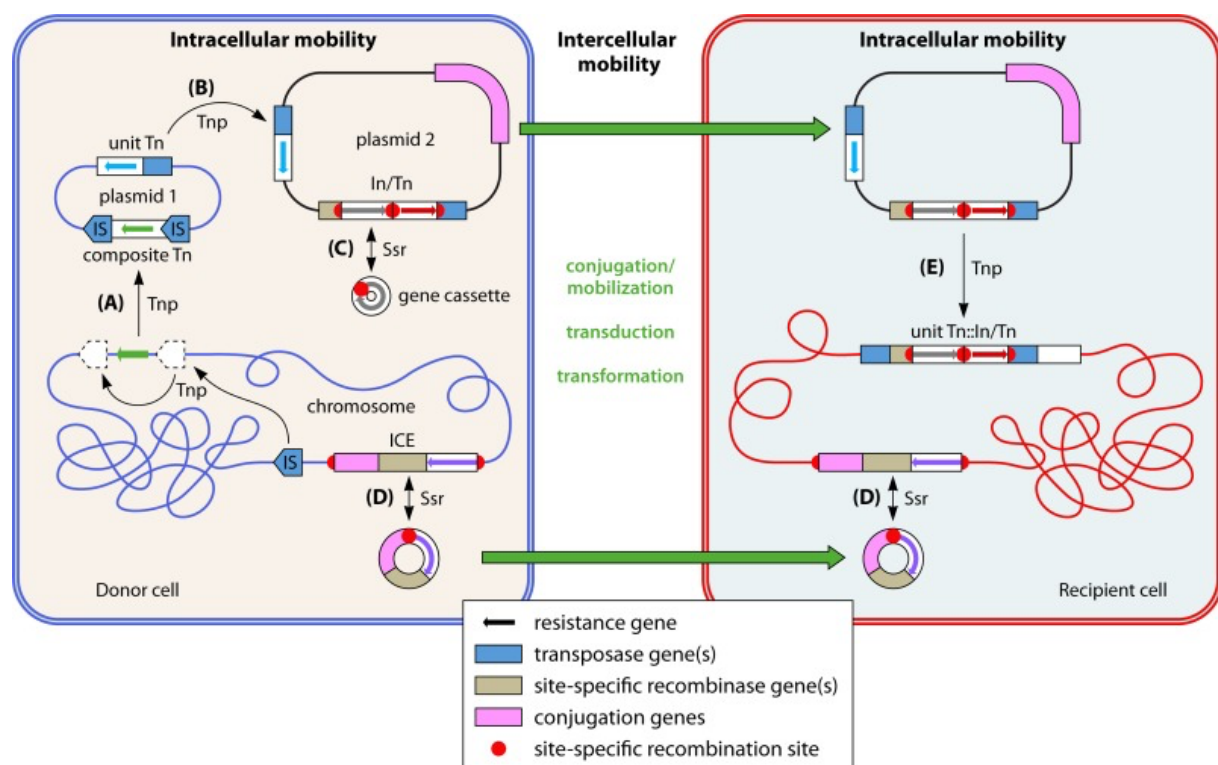


Figure 5. Overview of MGEs and intra- & extracellular mobility

Two different cells are depicted with examples of intracellular mobility inside the cells and intercellular mobility between the donor cell (blue) and the recipient cell (red) symbolised by green arrows. Thin black arrows denominate intracellular processes either mediated by a transposase (Tnp) or a site-specific recombinase (Ssr).

(A) Translocation of a composite transposon (framed by two insertion sequences) from chromosome to plasmid via a Tnp

(B) Translocation of a unit transposon between two plasmids via a Tnp

(C) integration of a gene cassette with a resistance gene into an integron/transposon via an integrase

(D) An ICE can be excised from the chromosome and is then available for HGT e.g. via conjugation. In the recipient cell it can be integrated back into the chromosome through a Ssr.

(E) The integron/transposon can translocate from plasmid to chromosome with a Tn.

(taken from Partridge et al.¹⁸⁸)

3.3.2. Genetic background of transferable MBLs

The majority of MBL genes, among them *bla*_{IMP}, *bla*_{VIM} and *bla*_{GIM}, are found as gene cassettes that are integrated into class 1 integrons, while *bla*_{IMP} can also be associated with class 3 integrons.^{138,166,225-227} Generally speaking, the integron is composed of three parts: the conserved 5' region, the variable region and the conserved 3' region. The 5' region consists of the integrase gene, recombination site and the promoter, while the MBL is part of the central variable region, where the integrase inserts the gene cassettes. Besides the MBL gene the variable region often contains ARGs conferring aminoglycoside-resistance, but also ARGs for other agents have been frequently described.²²⁸ By 2006, more than 80 distinct gene cassettes had been found in class 1 integrons.²²³ The 3' region in these integrons is most often composed by a *qacEΔ1*- and a *sul*-gene, which mediate resistance to antiseptics and sulfonamides, respectively.^{139,140,229} Class 1 integrons are generally thought to be associated to Tn402-like transposons and the Tn3-family, which could represent a keystone in their dissemination.²³⁰ MBLs of the NDM-type on the other hand, appear to not be associated with integrons. *bla*_{NDM} has been found widely dispersed in *Acinetobacter* isolates, where it is often associated with a Tn125 transposon formed out of two copies of *ISAb*125.^{157,160,231-233} Inside of this composite transposon a *ble*_{MBL}- and a truncated *trpF*-gene have been frequently found downstream of the MBL, that encode BRP_{MBL}, a protein responsible for bleomycin-resistance and a N-(5'-phosphoribosyl) anthranilate isomerase, respectively.^{233,234}

3.4. Aim of the study

So far, little research on carbapenem-resistance in *A. pittii* has been conducted because of the clinical predominance of *A. baumannii* infections. Hence, there are few data available on class B carbapenemases in *A. pittii* and on their genetic background. The aim of this study was to examine and characterise the genetic platform (i.e., the genetic location, e.g., chromosomally or plasmid-encoded genes) of MBLs in clinically unrelated *A. pittii* isolates by whole genome sequencing. As MBLs are mainly found on transferable plasmids in *Enterobacterales* and in *A. baumannii*²³⁵, we hypothesized that the MBLs present in the investigated *A. pittii* isolates were also located on plasmids. Furthermore, we intended to fully analyse MBL-harbouring plasmids and to compare them to annotated plasmids from the GenBank database.

4. Material and Methods/Results

Since the thesis at hand is presented in form of a cumulative dissertation, material and methods as well as the results will be presented in form of the scientific publication “Carbapenem resistance in *Acinetobacter pittii* isolates mediated by metallo- β -lactamases”, where these sections are described in their entirety.²³⁶ In the course of this work 17 MBL-carrying *A. pittii* from Germany, collected between 2010 and 2015 by the National Reference Center for Gram-negative hospital pathogens in Bochum, were examined. The genetic platform was assessed through S1-PFGE and Southern blot hybridisation. In isolates where plasmid-borne MBLs were detected, closed plasmid sequences were obtained using short- and long-read sequencing and hybrid-assemblies. The plasmid sequences were then annotated, visualized and compared to plasmids already submitted to the GenBank database provided by NCBI. Conjugation of MBL-carrying plasmids was tested. All 17 isolates alongside the obtained transconjugants were tested for antimicrobial susceptibility to a variety of antimicrobial agents. *bla*_{NDM}-harbouring plasmids were similar to previously described plasmids found all over the world, while *bla*_{GIM}- and *bla*_{VIM}-harbouring plasmids were novel. All MBLs were associated with MGEs. *bla*_{GIM} and *bla*_{VIM} were embedded in an integron, which showed high similarity to a previously described unit transposon. *bla*_{NDM} was shown to be associated with IS-elements.

The data provides a novel insight into the genetic background of MBLs in *A. pittii* and their distribution through HGT and MGE.

My contribution to the following publication were the conduction of S1-PFGE, Southern blot, antimicrobial susceptibility testing, conjugation experiments, long-read sequencing (partially), data interpretation and writing of the manuscript.

Carbapenem resistance in *Acinetobacter pittii* isolates mediated by metallo- β -lactamases

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Objectives: To characterize the genetic environment of metallo- β -lactamases (MBL) in carbapenem-resistant clinical *Acinetobacter pittii* isolates.

Methods: Seventeen carbapenem-resistant *A. pittii* isolates harbouring an MBL were collected between 2010 and 2015 in Germany. Antimicrobial susceptibility testing was performed using agar dilution. Presence of MBLs was confirmed by PCR and their genetic location determined by S1-pulsed-field gel electrophoresis followed by Southern blot hybridization. Whole-genome sequencing was performed using the Miseq and MinION platforms. Isolates were typed using an *ad hoc* core genome MLST scheme. Conjugation into *A. baumannii* was tested by broth mating.

Results: In 10 isolates the MBL was plasmid-encoded and in seven isolates chromosomally encoded. *bla*_{GIM-1} and *bla*_{VIM-2} were plasmid-encoded, *bla*_{VIM-4} was chromosomally encoded, while *bla*_{NDM-1} was chromosomally encoded in four and plasmid-encoded in three isolates. Seven of ten plasmids were conjugative into *A. baumannii*. Although most isolates were unrelated, the backbones of the MBL-encoding plasmid showed >99% similarity and only differed in the MBL-encoding area. *bla*_{NDM-1}-harbouring plasmids were highly similar to other plasmids from *Acinetobacter* isolates worldwide while the *bla*_{VIM-2}- and *bla*_{GIM-1}-encoding plasmids have not been described.

Conclusions: These data show the existence of a promiscuous plasmid circulating in *A. pittii* isolates in Germany that differs only in the MBL-encoding region. Its plasmid backbone has been found globally among multiple *Acinetobacter* spp. These data should raise awareness of an epidemic conjugative plasmid that has independently acquired MBLs. We should also consider that future comparative plasmid analysis will look beyond solely the resistome and include the mobile elements carrying the resistance genes.

Introduction

The genus *Acinetobacter* comprises a heterogeneous group of non-fermentative, oxidase-negative, Gram-negative bacteria. Its most important representative, *Acinetobacter baumannii*, is a leading cause of nosocomial infections, e.g. nosocomial pneumonia, bloodstream infections, wound infections, urinary tract infections and meningitis. The treatment of infections caused by *A. baumannii* poses a challenge because of their frequent resistance to various antimicrobials including carbapenems.¹

Acinetobacter species other than *A. baumannii* are equally involved in nosocomial infections, particularly those species that belong to the so-called 'A. baumannii group'.² This group

comprises five of the clinically most relevant *Acinetobacter* species, i.e. *A. baumannii*, *A. pittii*, *A. nosocomialis*, *A. seifertii* and *A. lactucae*, which are grouped together based on phenotypic and genotypic similarity as well as clinical relevance.^{1,3–6}

A. pittii is frequently found as a commensal on human skin and mucous membranes, but there are reports of it being associated with bloodstream infections, occasionally causing epidemic outbreaks in a clinical setting.^{7,8} Thus, the emergence of carbapenem resistance in *A. pittii* is of particular concern, as carbapenems are considered last resort antibiotics to treat infections caused by multi-drug resistant Gram-negative bacteria. Carbapenem resistance in *A. pittii* (CRAp) is often

mediated by class D carbapenemases such as OXA-23, OXA-40 and OXA-58, or by class B metallo- β -lactamases such as GIM, IMP, NDM or VIM.^{9–11}

The New-Delhi metallo-beta-lactamase (NDM) was first detected in 2008 in a *Klebsiella pneumoniae* isolate.¹² Since then, it has been reported in many Gram-negative species from more than 40 countries, some of which predate 2008.¹³ Diverse plasmids harbouring *bla*_{NDM} have been described, showing the ability for transmission to other bacterial species and genera.¹³

Although the spread of NDM was initially thought to be epidemiologically associated with the Indian subcontinent,¹⁴ *bla*_{NDM-1} has also been identified in *A. baumannii* isolates from patients from Eastern Europe the Middle East and North Africa.^{15,16} In *A. pittii*, *bla*_{NDM-1} is now found widespread in Asia, the Middle East, as well as Europe and South America.^{17–20} The first discovery of the Verona imipenemase (VIM-1) was in 1999 in Verona in a *Pseudomonas aeruginosa* isolate.²¹ The variants VIM-2 and VIM-4 are highly similar to VIM-1²² but have so far been rarely found in *Acinetobacter*.^{23,24} *bla*_{VIM-2} has so far only been described twice in *A. pittii*, in one isolate from Poland,²⁵ where it was plasmid-encoded, and in another from Korea but the location of the gene was not investigated.²⁶ The first German imipenemase (GIM-1) was discovered in five *P. aeruginosa* isolates from Germany in 2002.²⁷ *bla*_{GIM-1} has so far been rarely documented in *Acinetobacter* spp. in Germany.²⁸ Kaase et al.⁹ have described a group of *A. pittii* isolates harbouring *bla*_{GIM-1}, of which five isolates are also included in our study.

As few data are available for *A. pittii* isolates harbouring the aforementioned carbapenemases, and few data exist on their genetic background, our aim in this study was to characterize the genetic platform of GIM-1, NDM-1, VIM-2 or VIM-4 in clinically unrelated CRAP isolates. As metallo-beta-lactamases (MBL) are mainly found on transferable plasmids in Enterobacterales and in *A. baumannii*,²⁹ we hypothesized that the MBLs present in the investigated *A. pittii* on plasmids.

Material and methods

Bacterial strain selection

Seventeen CRAP isolates from the German National Reference Laboratory for Gram-negative organisms, as well as from the collection of isolates at the Institute for Medical Microbiology, Immunology and Hygiene at the University of Cologne obtained between 2010 and 2015 were investigated. The isolates originated from different cities in Germany. The species were identified by *gyrB* multiplex PCR³⁰ and confirmed using the whole-genome sequences at JSpecies (<https://jspecies.ribohost.com/jspeciesws/>).

Antimicrobial susceptibility testing

Susceptibility to gentamicin, trimethoprim:sulfamethoxazole (19:1), tetracycline (all from Sigma-Aldrich, Steinheim, Germany); amikacin, ciprofloxacin, imipenem and meropenem (all from Molekula, Newcastle-upon-Tyne, UK) was determined using agar dilution method following the current CLSI guidelines.³¹ All susceptibility tests were repeated at least three times independently. *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. MICs were interpreted using the resistance breakpoints for *Acinetobacter* spp. of the M100 Guideline from CLSI.³²

Whole-genome sequencing and core genome MLST (cgMLST)

Total DNA was extracted from overnight cultures using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH, Munich, Germany) for a 250-bp paired-end sequencing run on an Illumina MiSeq. The resulting FASTQ files were assembled *de novo* using the Velvet assembler that is integrated in Ridom SeqSphere⁺ v.7.0.3 software (Ridom GmbH, Münster, Germany).³³ *In silico* detection of resistance genes was performed using ResFinder v.4.1 and CARD v.3.1.4.^{34,35} Pasteur sequence types were determined through PubMLST.³⁶ An *ad hoc* cgMLST scheme was created using Ridom SeqSphere⁺ v.7.0.3 software based on 2821 alleles using the *A. pittii* PHEA-2 genome.³⁷

Determination of MBL gene location

To assess the genetic platform of *bla*_{GIM-1}, *bla*_{NDM-1}, *bla*_{VIM-2} and *bla*_{VIM-4} as well as the approximate size of plasmids, S1-nuclease digestion followed by pulsed-field gel electrophoresis (S1-PFGE) and Southern blot hybridization were performed.

S1-PFGE was conducted as previously described by Xanthopoulou et al.³⁸ The location of MBL-encoding genes was visualized by performing Southern blot hybridization with digoxigenin-labelled probes (Roche, Mannheim, Germany). Signal detection was performed following the manufacturer's instructions using CDP-Star[®] ready-to-use (Roche) chemiluminescent substrate and visualized on the ChemiDoc MP Imaging System (BIO-Rad, Munich, Germany). Chromosomal location was shown by co-localization with a *gyrB*-probe.

Long-read sequencing and bioinformatics

In 10 isolates where the MBL was shown to be plasmid located, long-read sequencing was conducted to obtain closed plasmids. DNA extraction, library preparation, MinION run and bioinformatic processing were conducted as previously described.³⁸ The long and the short reads were assembled using the Unicycler assembly pipeline.³⁹ Genome and plasmid annotation was conducted using RAST^{40,41} and manually checked. IS-elements and transposons were identified using ISfinder (<https://isfinder.biotoul.fr/>). The *oriT* region was identified using oriTfinder (<https://bioinfo-mml.sjtu.edu.cn/oriTfinder/>).

Conjugation experiments

In the 10 isolates where the MBL was shown to be plasmid-encoded, conjugation experiments were performed by broth mating with the sodium-azide resistant *E. coli* J53 as well as the rifampicin resistant *A. baumannii* BM4547 as recipients. Selection of transconjugants was performed using meropenem and 200 mg/L sodium azide (*E. coli* J53) or meropenem and 50 mg/L rifampicin (*A. baumannii* BM4547). The meropenem concentration used reflected the MICs of the donor strains; 4 mg/L for UKK-0556, UKK-0555, UKK-0554, UKK-0553 and UKK-0265; 16 mg/L for UKK-0547 and UKK-0548, and 32 mg/L for UKK-0540, UKK-0539 and UKK-0538. An additional selection for transconjugants into J53 was performed using a lower meropenem concentration of 1 mg/L.

Transconjugants were confirmed by multiplex-PCR for the presence of the plasmid-encoded MBL and by *gyrB* multiplex-PCR to differentiate between donor (*A. pittii*) and recipient (*A. baumannii*).^{30,42} Antimicrobial susceptibilities of the seven transconjugants and the recipient BM4547 were determined by agar dilution as described before.

Access to data

The raw sequencing reads generated in this project, genome and plasmid assemblies are available at NCBI under the BioProject accession number

PRJNA322121. The long reads are submitted under the BioProject number PRJNA853156.

Results

Strain selection and resistome

The 17 *A. pittii* isolates were confirmed to harbour MBL genes using whole-genome sequencing. Five isolates carried *bla*_{GIM-1}, seven isolates *bla*_{NDM-1}, two isolates *bla*_{VIM-2} and three isolates *bla*_{VIM-4} (Table 1). Furthermore, all isolates also harboured a variety of different antibiotic resistance genes. Using seven-loci MLST, we found a wide variety of sequence types (STs), but also some (ST63, ST119, ST248) that were found multiple times. Finetyping using the *ad hoc* cgMLST scheme revealed that most isolates were unrelated despite having the same ST, apart from the three isolates with chromosomally encoded *bla*_{NDM-1} (UKK-0541 to –0543) that clustered closely together (Figure 1). Apart from this cluster, there was no correlation between ST and resistome.

Antimicrobial susceptibility

The results of the antimicrobial susceptibility testing are summarized in Table 1. All isolates were resistant to the carbapenems tested, with only the GIM-positive UKK-0556 and UKK-0555 being intermediate to imipenem. Additionally, like most *Acinetobacter* all isolates were resistant to ceftazidime with MICs of 32 µg/mL and higher. The four isolates carrying chromosomally encoded *bla*_{NDM-1} were susceptible to both tested aminoglycosides, while the three isolates harbouring plasmid-encoded *bla*_{NDM-1} were susceptible to gentamicin and showed intermediate susceptibility to amikacin. The five isolates harbouring *bla*_{GIM-1} were all found to be non-susceptible to gentamicin and amikacin. The isolates with *bla*_{VIM-2} had high MIC values for both aminoglycosides. The three isolates encoding *bla*_{VIM-4} showed resistance to gentamicin but remained susceptible to amikacin. Fluoroquinolone resistance was found in five isolates and was associated with substitution in GyrA and ParC (Supplementary data 1, available as Supplementary data at JAC online). Tetracycline resistance was only observed in UKK-0548 which harboured *tet*(39) on plasmid p5_UKK-0548. Resistance to trimethoprim:sulfamethoxazole was present in all

Table 1. Overview of *A. pittii* isolates with year, city (where known) and state in Germany, Pasteur ST, resistome and antimicrobial susceptibility testing results (MIC values)

Sample	Year	Origin	ST	MBL	Other resistance genes	MIC (µg/mL)						
						MEM	IPM	GEN	AMK	CIP	TET	SXT
UKK-0265	2012	Leverkusen, NW	205	GIM-1	<i>ant</i> (2'')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-70} , <i>bla</i> _{OXA-417} , <i>sul</i> 1	16	8	8	32	0.125	2	152/8
UKK-0553	2011	Dresden, SN	63		<i>ant</i> (2'')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>sul</i> 1	8	8	16	32	0.5	2	304/16
UKK-0554	2011	Bochum, NW	249			32	16	8	32	16	1	76/4
UKK-0555	2011	NW	93			32	4	8	32	0.25	2	304/16
UKK-0556	2010	Bochum, NW	248			8	4	64	64	64	2	152/8
UKK-0536	2014	Cologne, NW	119	NDM-1	<i>bla</i> _{ADC-150} , <i>bla</i> _{OXA-421} , <i>ble</i> _{MBL}	128	128	2	4	0.5	4	9.5/0.5
UKK-0538	2011	Cottbus, BB	63		<i>aph</i> (3')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>ble</i> _{MBL}	256	256	2	32	8	4	4.75/0.25
UKK-0539	2013	Berlin, BE	63		<i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>ble</i> _{MBL}	256	256	1	32	0.25	1	9.5/0.5
UKK-0540	2014	Schwerin, MV	778		<i>aph</i> (3')-VI, <i>bla</i> _{ADC-22-like} , <i>bla</i> _{OXA-501} , <i>ble</i> _{MBL}	256	256	2	32	0.5	2	9.5/0.5
UKK-0541	2015	Berlin, BE	63		<i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>ble</i> _{MBL}	256	256	1	4	0.25	1	9.5/0.5
UKK-0542	2015	Berlin, BE	63			256	256	1	4	0.25	1	4.75/0.25
UKK-0543	2015	Berlin, BE	63			128	128	1	4	0.25	1	4.75/0.25
UKK-0547	2012	Dresden, SN	63	VIM-2	<i>aac</i> (6')-Ib-like, <i>ant</i> (2'')-Ia-like, <i>aph</i> (3')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>sul</i> 1	64	256	>256	128	0.5	4	304/16
UKK-0548	2013	SN	248		<i>aac</i> (6')-Ib-like, <i>aph</i> (3')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>sul</i> 1, <i>tet</i> (39)	64	256	>256	128	64	128	304/16
UKK-0549	2012	SN	746	VIM-4	<i>aac</i> (6')-Ib, <i>ant</i> (2'')-Ia, <i>aph</i> (3')-Ia, <i>bla</i> _{ADC-18} , <i>bla</i> _{CARB-16} , <i>bla</i> _{OXA-500} , <i>sul</i> 1	64	64	64	8	0.5	2	152/8
UKK-0550	2013	TH	119		<i>aac</i> (6')Ib, <i>ant</i> (2'')-Ia, <i>aph</i> (3')-Ia, <i>strA</i> , <i>strB</i> , <i>bla</i> _{ADC-150} , <i>bla</i> _{CARB-16} , <i>bla</i> _{OXA-421} , <i>sul</i> 1, <i>sul</i> 2	32	64	32	8	0.5	2	304/16
UKK-0551	2014	SN	63		<i>aac</i> (6')-Ib-like, <i>ant</i> (2'')-Ia-like, <i>aph</i> (3')-Ia, <i>aph</i> (3')-VI, <i>bla</i> _{ADC-18} , <i>bla</i> _{OXA-500} , <i>sul</i> 1	16	64	32	4	0.5	2	76/4

BB, Brandenburg; BE, Berlin; MV, Mecklenburg-Western Pomerania; NW, Northrhine-Westphalia; SN, Saxony; TH, Thuringia; MEM, meropenem; IMP, imipenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; TET, tetracycline; SXT, trimethoprim:sulfamethoxazole (19:1).

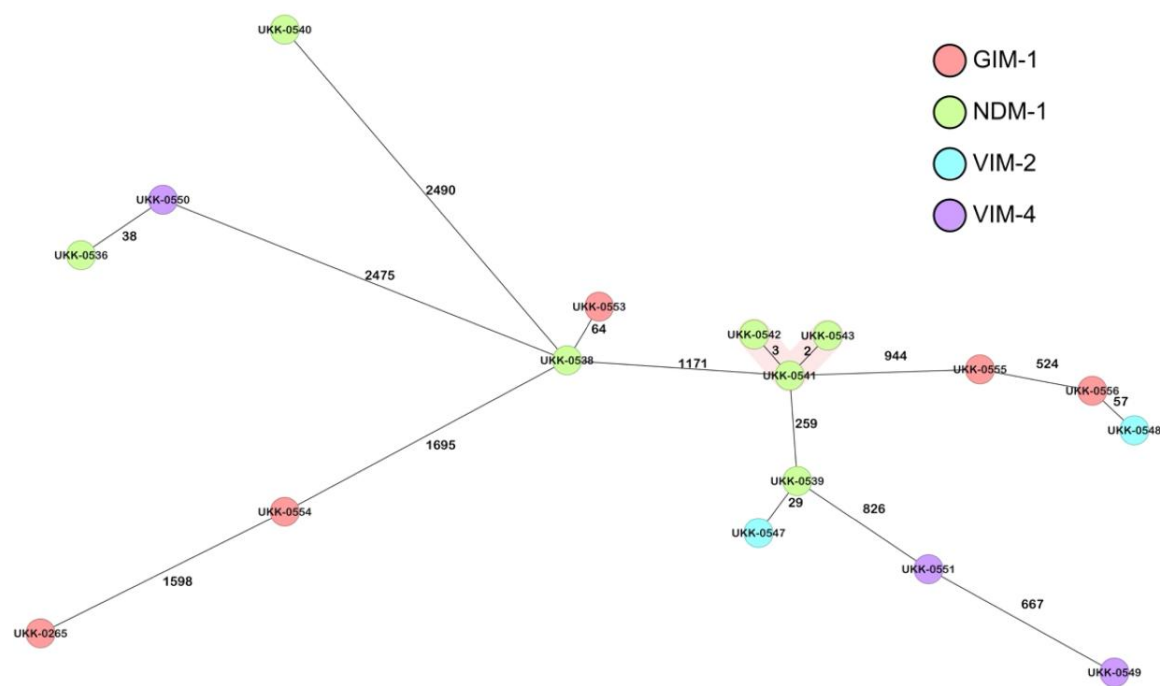


Figure 1. Minimum spanning tree generated using Ridom SeqSphere⁺ for 17 samples based on 2821 alleles, pairwise ignoring missing values. Distance based on columns from *A. pittii* PHEA-2. Isolates are coloured according to harboured MBL. The numbers between the nodes indicate the number of alleles different between the two isolates. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

isolates harbouring either *bla*_{GIM-1}, *bla*_{VIM-2} or *bla*_{VIM-4} and not in those carrying *bla*_{NDM-1} and aligned with the presence of *sul1* or *sul2*.

Genetic platform of MBLs

Southern hybridization results revealed that the MBL was plasmid-encoded in 10 isolates and chromosomally encoded in the remaining seven (Table 2). *bla*_{GIM-1} and *bla*_{VIM-2} were located on a plasmid, whereas *bla*_{VIM-4} was chromosomally encoded. In three isolates *bla*_{NDM-1} was plasmid-encoded, and chromosomally located in the other four. Plasmids harbouring the same MBL were shown to be similar in size.

Conjugation and transconjugant antimicrobial susceptibility

Out of 10 MBL-encoding plasmids, seven were conjugative into the *A. baumannii* recipient BM4547 (Table 2). There were no transconjugants into the *E. coli* recipient J53. In the transconjugants, we saw elevated MICs to carbapenems in comparison to BM4547, confirming that carbapenem resistance was mediated through the MBLs encoded on the plasmids (Table 3). MICs to ceftazidime was also increased in the obtained transconjugants, showing that resistance to cephalosporins is not only caused by the chromosomally encoded *bla*_{ADC}-variant, but also by the MBL. Aminoglycoside and trimethoprim:sulfamethoxazole (19:1) MICs were only increased in those transconjugants where the donor strain was also resistant. Neither ciprofloxacin nor tetracycline MICs increased.

Table 2. Overview of isolates with encoded MBL, gene location and plasmid size and conjugativeness in plasmid-encoded *A. pittii* isolates

Isolate	MBL	MBL gene location	Plasmid size (bp)	Conjugative
UKK-0265	GIM-1	plasmid	39 562	No
UKK-0553			43 536	Yes
UKK-0554			42 583	Yes
UKK-0555			42 583	Yes
UKK-0556			43 772	Yes
UKK-0538	NDM-1	plasmid	47 278	Yes
UKK-0539			49 842	Yes
UKK-0540			46 164	Yes
UKK-0536		chromosome	N/A	N/A
UKK-0541				
UKK-0542				
UKK-0543				
UKK-0547	VIM-2	plasmid	46 530	No
UKK-0548			45 234	No
UKK-0549	VIM-4	chromosome	N/A	N/A
UKK-0550				
UKK-0551				

N/A, not applicable.

Analysis of *bla*_{GIM-1} environment

All five plasmids harbouring *bla*_{GIM-1} were shown to be highly similar (Supplementary data 2). In all isolates the resistance

Table 3. Antimicrobial susceptibility testing results (MIC values) determined through agar dilution of transconjugants and recipient strain

Isolate	MIC (µg/mL)							
	MEM	IPM	CAZ	GEN	AMK	CIP	TET	SXT
BM4547	1	0.125	8	8	8	1	8	2.375/0.125
BM4547:: pGIM1_UKK-0553	64	16	256	32	32	2	8	76/4
BM4547:: pGIM1_UKK-0554	64	16	>256	64	32	2	8	76/4
BM4547:: pGIM1_UKK-0555	64	16	>256	32	32	1	8	76/4
BM4547:: pGIM1_UKK-0556	32	16	256	32	32	1	8	76/4
BM4547:: pNDM1_UKK-0538	>256	256	>256	4	32	1	8	2.375/0.125
BM4547:: pNDM1_UKK-0539	>256	256	>256	8	32	1	8	2.375/0.125
BM4547:: pNDM1_UKK-0540	>256	256	>256	8	32	1	8	2.375/0.125

MEM, meropenem; IMP, imipenem; CAZ, ceftazidime; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; TET, tetracycline; SXT, trimethoprim:sulfa-methoxazole (19:1).



Figure 2. Overview of genetic surroundings of *bla*_{GIM-1} (a), *bla*_{VIM-2} (b), *bla*_{VIM-4} (c) and *bla*_{NDM-1} (d). Arrows represent predicted ORFs, and the direction of the arrow denotes the direction of transcription. Hypothetical proteins are not shown. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

genes *sul1*, *ant(2'')-Ia* and *bla*_{GIM-1} as well as the *qacEΔ1* efflux pump were grouped in an integron with genes encoding for an *N*-acetyltransferase and an *IntI1*-like integrase. The surroundings of the MBL-encoding genes were identical in all isolates and showed similarity to the transposon TnAs3 [Figure 2(a)].

The genes encoding for the *N*-acetyltransferase, *sul1* as well as the *qacEΔ1* are all part of TnAs3 while *ant(2'')-Ia* and *bla*_{GIM-1} are not. The integrase found next to *bla*_{GIM-1} was 95% identical to *IntI1* from TnAs3. Resistance gene *aph(3')-VI* was located between the IS-element *ISAb14* and *tnpR*. While

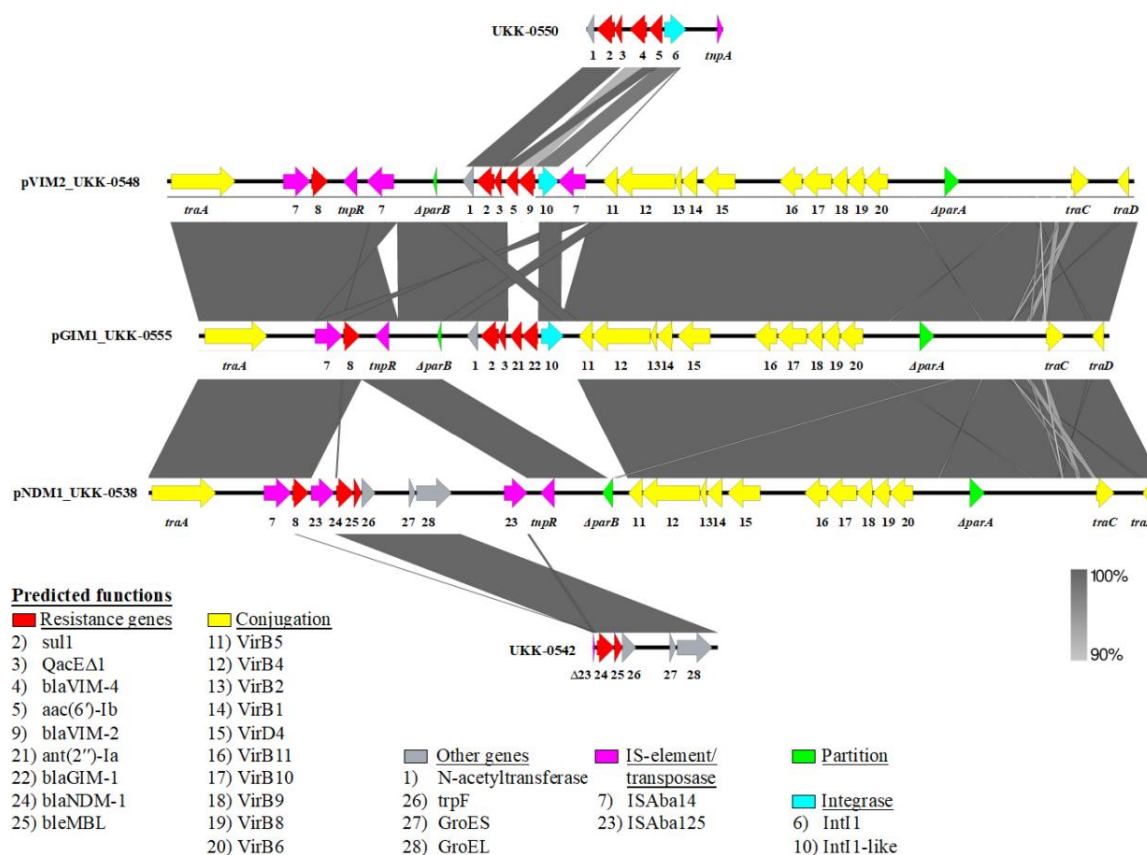


Figure 3. Comparison of *bla*_{VIM-2}-harbouring plasmid pVIM2_UKK-0548, *bla*_{GIM-1}-harbouring plasmid pGIM1_UKK-0555, *bla*_{NDM-1}-harbouring plasmid pNDM1_UKK-0538 and genetic surroundings of *bla*_{VIM-4} and *bla*_{NDM-1} in UKK-0550 and UKK-0542, respectively. Regions of identity between nucleotide sequences are represented by grey bars. The cut-off value for identity is 90% across a region of 50 nucleotides. The figure was generated using EasyFig_2.2.2 (<https://mjsull.github.io/Easyfig/>). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

pGIM1_UKK-0555 and pGIM1_UKK-0554 were identical, pGIM1_UKK-0265 was missing 3019 bp that harboured genes of the VirB type IV secretion system (T4SS): *virB8* and *virB9*, with *virB6* and *virB10* only being partially encoded. In the other four *bla*_{GIM-1}-harbouring plasmids, 10 genes encoding for proteins from the T4SS could be found, namely *virB1*, *virB2*, *virB4*–6, *virB8*–11 and *virD4*. All isolates also harboured *traA*, *traC* and *traD* genes as well as *AparA* and *AparB*. In comparison to pGIM1_UKK-0555, pGIM1_UKK-0553 had an additional insertion sequence next to *aph(3')-VI* (*ISAlw15*), while in plasmid pGIM1_UKK-0556 we found an additional *ISAbA1* between the *AparB* and the *N*-acetyltransferase encoding genes, which was bracketed by target site duplications of 9 bp. Apart from these IS-elements pGIM1_UKK-0556 and pGIM1_UKK-0553 showed 100% identity to both pGIM1_UKK-0555 and pGIM1_UKK-0554.

Analysis of *bla*_{VIM-2} environment

Both plasmids harbouring *bla*_{VIM-2} were highly similar to each other except for an additional *ISAbA14* found in pVIM2_UKK-0548 close to the integron and two *ISAcsp5* in pVIM2_UKK-0547, one disrupting the other (Supplementary data 3). The disrupted *ISAcsp5* was framed by target site

duplications of 4 bp. The integron harbouring *bla*_{VIM-2}, *aac(6')-Ib*, *qacEΔ1*, *sulI* and the *N*-acetyltransferase is very similar to the integron found in the five plasmids harbouring *bla*_{GIM-1} and differs only in the MBL and the aminoglycoside-resistance gene [Figure 2(b)]. Resistance gene *aph(3')-VI* was again found next to *tnpR* and bracketed by two identical *ISAbA14* elements. The other genes found in this plasmid were identical to those found in the five plasmids harbouring *bla*_{GIM-1}.

Analysis of *bla*_{NDM-1} environment

The genetic surroundings of *bla*_{NDM-1} were similar in all isolates, despite four being chromosomally and three plasmid-encoded. Downstream of *bla*_{NDM-1}, *bleMBL* and *trpF* were found. Upstream of the MBL we detected *ISAbA125* in all isolates. In the four isolates that harboured the plasmid-encoded MBL, we also found *aph(3')-VI* as well as an *ISAbA14* upstream from the MBL [Figure 2(d)]. All three plasmids harbouring *bla*_{NDM-1} were highly similar to each other (identity >99.9%) and only differed significantly in additional IS-elements (Supplementary data 4). Compared to pNDM1_UKK-0540, both pNDM1_UKK-0538 and pNDM1_UKK-0539 harboured an additional *ISAbA125* located close to *tnpR*. The thus formed transposon formed by the two

ISAb125 was bracketed by target site duplications of 3 bp. pNDM1_UKK-0539 harboured an additional ISAb14 compared to pNDM1_UKK-0538, which disrupted the ISAb125 located upstream from the MBL and displayed target site duplications of 3 bp. In this plasmid, an additional ISAb-14 was also found close to *AprA*. Apart from these genes, we found the same VirB type IV transport complex encoding genes that were also found in all other MBL-harboring plasmids in this study, as well as *AprA*, *AprB*, *traA*, *traC* and *traD* genes. Additionally, there were TnAs3, ISAb125 and *tnpR* found next to *groEL* and *groES* genes.

Analysis of bla_{VIM-4} environment

The genetic surroundings of chromosomally encoded bla_{VIM-4} are highly similar in all three isolates, with more than 99.95% identity (Supplementary data 5). The integron contains an integrase (*intI1*), *aac(6')-Ib*, bla_{VIM-4}, *qacE11*, *sul1* and a gene encoding for a N-acetyltransferase and is highly similar to the integron found in the groups harbouring bla_{GIM-1} and bla_{VIM-2} [Figure 2(c)]. Except for *aac(6')-Ib* and bla_{VIM-4}, the integron is again highly similar to the TnAs3-transposon. A major part of the integron, which only lacked a small fragment of the N-acetyltransferase gene, could also be found on the chromosome of *K. pneumoniae* strain 51135CZ (Acc. no. CP085740.1) as well as two plasmids found in two *Citrobacter* isolates: p56415CZ_VIM (Acc. no. CP085728.1) and p51929_MCR_VIM (Acc. no. CP059429.1).

Comparative analysis between plasmids

Despite harbouring different MBLs, all 10 plasmids were found to be highly similar. They were of similar size (39.5–49.8 kbp) and shared the same backbone harbouring the same genes and only differed in the MBL-encoding region (Figure 3). bla_{VIM-2} and bla_{GIM-1} were located in an integron with *IntI1*-like and a variable set of other resistance genes, while bla_{NDM-1} was not associated with an integrase but bracketed by two copies of ISAb125 in pNDM1_UKK-0538 and pNDM1_UKK-0539 and located close to one copy of this IS-element in pNDM1_UKK-0540. A BLAST search of the plasmid backbone showed a variety of highly similar plasmids, which were only found in *Acinetobacter* isolates and not in other species.

Analysis of other resistance gene-harboring plasmids

In addition to the MBL-harboring plasmids, five other plasmids harbouring other resistance genes were found. Three of these plasmids, i.e. p4_UKK-0538, p7_UKK-0548 and p5_UKK-0547, were identical, had a size of 8804 bp and harboured the resistance gene *aph(3')-Ia* (Supplementary data 6). This plasmid has so far not been described and most closely resembles the cloning vector pBRH1 (Acc. no. Y14439.1); 56% of the plasmid align with pBRH1 with an identity of more than 99% including *aph(3')-Ia*, *Is903*, *pBBR1*, the *mob* protein and the *catA1*. The 11,025 bp p5_UKK-0548 harboured *tet(39)* alongside a replication protein (*repB*) and an integrase (Supplementary data 7). This plasmid has so far not been described and is most similar to unnamed plasmid 6 from the *A. johnsonii* isolate FDAARGOS_1094 (Acc. no. CP068191.1). The plasmids share a sequence of almost 4700 bp including the integrase and an unknown type IV secretory system with an identity of roughly 95%. The 6078 bp plasmid p7_UKK-0547 harboured *ant(2'')-Ia* alongside both *mobA*-

and *mobC*-genes (Supplementary data 8). It only differed from plasmid pR32_3 by 1 bp (Acc. no. CP091597.1).

Discussion

In this study we investigated carbapenem resistance in *A. pittii* mediated by MBLs and in particular the genetic platform of plasmid-encoded resistance determinants. Non-susceptibility to meropenem and imipenem was attributed to the presence of an MBL, which was further supported by the fact that resistance to these agents was transferred into conjugation recipient strain BM4547. While in most isolates the genotypes and phenotypes aligned concerning the resistance to aminoglycosides, there were a few inconsistencies. In isolate UKK-0548, which was resistant to both aminoglycosides, we could not find a gene responsible for gentamicin resistance. Furthermore, UKK-0549, UKK-0550 and UKK-0551 were susceptible to amikacin, despite harbouring *aac(6')Ib* which normally mediates amikacin-resistance.⁴³ Further investigation into aminoglycoside-resistance mechanisms in the *Acinetobacter* genus might clarify their underlying resistance mechanisms. Fluoroquinolone resistance was associated with mutations in *gyrA*- and *parC*-genes as this is the main resistance mechanism known for *A. baumannii*.^{44,45} In all five ciprofloxacin-resistant isolates, a Ser81-Leu substitution was found in *GyrA*. UKK-0554, which showed a marginally higher MIC of 16 µg/mL, had an additional Ser84-Leu substitution in *ParC*. Higher MICs of 64 µg/mL were associated with an additional Glu88-Lys substitution in *ParC* (Supplementary data 8). *GyrA* and *ParC* substitutions were the same as those reported in *A. baumannii* but were in the past numbered according to *E. coli* (Ser83-Leu in *GyrA* and Ser80-Leu and Glu84-Lys in *ParC*).^{44,45}

It has been previously reported that the VirB/VirD4 T4SS consists of 12 genes in total that are essential for plasmid conjugation.⁴⁶ However, all plasmids that were examined harboured only 10 of the genes encoding for the VirB/VirD4 system with *virB3* and *virB7* not being encoded on any plasmid. Nonetheless, seven plasmids were shown to be conjugative. Considering that in other species there are other T4SSs which consist of fewer than these 12 genes, for example in streptococci,^{47,48} we conclude that *virB3* and *virB7* are not necessary for conjugation in *A. pittii*. pGIM1_UKK-0265 was not conjugative as it had an incomplete *virB6* and *virB8-10*, but was otherwise completely identical to conjugative plasmid pGIM1_UKK-0555. This shows that these proteins play an important role in conjugation in *A. pittii*, too. However, pVIM2_UKK-0548 and pVIM2_UKK-0547 were not conjugative despite harbouring the same 10 genes from the T4SS-complex. This indicates that there are other unknown determinants that also play a role in conjugation, that are not present on the other two plasmids. Further investigations could support these findings and identify the unknown element(s).

Despite harbouring different MBLs, the backbones of all 10 plasmids were shown to be highly similar. The plasmids were of similar sizes and shared the same backbone, and only significantly differed in the MBL-encoding region. The first bla_{NDM-1}-carrying plasmids that were characterized in *Acinetobacter* spp. were pNDM-BJ01 and a close variant, pNDM-BJ02, found in *A. lwoffii* isolates from China.⁴⁹ We found a 99.9% identity between pNDM-BJ01 and pNDM1_UKK-0538, as well as pNDM-BJ02 and pNDM1_UKK-0540, which suggests that the plasmids in this

study are derivatives of these plasmids, but we are unaware of any epidemiological link between China and our isolates. Plasmids found in *Acinetobacter* species generally tend to harbour a replicase; however, like pNDM-BJ-01 and its derivatives the MBL-harbouring plasmids of our isolates did not harbour any known replicase.⁵⁰ The three *bla*_{NDM-1}-harbouring plasmids found in our isolates also share significant similarity to a multitude of other plasmids from North and South America, the Middle East and Asia, with identities of more than 99.9%. pAhaeAN54e (Acc. no. CP041229.1) came from a Mexican *A. baumannii* isolate, p6200-47274kb (Acc. no. CP010399.1), was also found in an *A. baumannii* isolate from Colombia, and pIEC383 (Acc. no. MK053932.1) was identified in a Brazilian *A. baumannii* isolate. pNDM-JVAP01 (Acc. no. KM923969.1) was obtained from a Turkish *A. lactucae* isolate. pNDM-40-1 (Acc. no. KF702385.1) was obtained from an *A. berezeniae* isolate from India while pNDM1_060092 (Acc. no. CP035935.1) derived from an *A. cumulans* isolate from China. The plasmids harbouring *bla*_{GIM-1} or *bla*_{VIM-2} harbour the same integrase, *IntI1*-like, which was not present in the plasmids harbouring *bla*_{NDM-1}. We suggest that this integrase is responsible for integrating the MBLs as well as other resistance genes into the respective plasmids. The fact that most of the resistance genes are grouped around the integrase supports this hypothesis. The integrase seems to derive from TnAs3 or a related transposon. BLAST analysis revealed that the *bla*_{VIM-2} or *bla*_{GIM-1}-harbouring plasmids from our study have not yet been described. As they were present in multiple unrelated isolates from all over the country, we hypothesize that this plasmid variant was already widespread in Germany in 2015. This promiscuous plasmid thus poses a potential threat to healthcare and efficacy of antimicrobial treatment. Interestingly enough the integron found in pVIM2_UKK-0547 and pVIM2_UKK-548 shows resemblance to the integron found in plasmids pFA267 (Acc. no. MT771289.1) and pFA648 (Acc. no. MT771290.1) found in *A. nosocomialis* as well as pFA319 (Acc. no. MT771291.1) found in *A. seifertii* from South Korea.²³ The integron in these plasmids also harbours an integrase, *bla*_{VIM-2}, *qacEΔ1*, *sul1* and the *N*-acetyltransferase-gene in the same order, but does not encode for *aac*(6')-Ib. Instead, *aacA7* and *aadA1* could be found in all three plasmids. Also, in pFA267 and pFA648 resistance gene *aph*(3')-VI could be found, which was also present in pVIM2_UKK-0547 and pVIM2_UKK-0548.

In summary, these data show new variants of a globally spread plasmid in *A. pittii* in Germany that have also acquired other resistance genes including MBLs. The source of the plasmid is unknown, although there are reports of similar plasmids worldwide in multiple *Acinetobacter* spp. This suggests that this plasmid could also be found in other *Acinetobacter* spp. in Germany. We therefore propose that the analysis of plasmids should not only concentrate on a single species, as mobile elements in *Acinetobacter* appear to have an inter-species range. Furthermore, we should consider looking for relatedness in plasmids harbouring different resistomes.

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Transparency declarations

The authors declare no conflict of interest.

Supplementary data

Available as [Supplementary data](#) at JAC online.

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5. Discussion

In recent years a worldwide emergence of MDR bacteria has been reported. The World Health Organisation (WHO) even goes as far as to consider antibiotic resistance “one of the biggest threats to global health, food security and development”.²³⁷ The presence of ARGs in bacterial wildtypes is frequent, but it is the common and frequent use, especially the overuse of antibiotics in healthcare and livestock settings, that provide the selective pressure for the rise of MDR phenotypes. Especially in *Acinetobacter* spp. MDR, XDR or PDR isolates are increasingly common because of their immense genome plasticity caused by their propensity for both inter- and intracellular gene transfer. The WHO has even named carbapenem-resistant *A. baumannii* a priority 1 pathogen for research and development of new antibiotic agents.²³⁸ Nonetheless the development of new antibiotic agents to counteract these trends is scarce.²³⁹ Carbapenems still constitute the gold standard in treatment of *Acinetobacter* infections in Germany. Because of their complex resistance profiles, treatment options are limited to last resort antibiotics in case of carbapenem resistance. In cases like these, AWMF (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften [translated by the author: Working committee of scientific medical professional societies]) guidelines suggest the use of colistin or tigecycline, often in combination with aminoglycosides or sulbactam in Germany.^{240,241}

Despite their pathogenic potential, research into carbapenem-resistant non-*baumannii* *Acinetobacter* spp. from the *A. baumannii* group is rare. Most research into MBL-producing *A. pittii* has been conducted with isolates from Asia^{161,242,243} or South America.^{76,162,244} There have, so far, been only few sporadic reports of European isolates in Poland¹⁵², in Turkey²⁴⁵, in Belgium²⁴⁶ and in Denmark.²⁴⁷ There were also two reports on GIM-1 producing *A. pittii* in Germany.^{169,170} Herrmann *et al.* have described a pseudo-outbreak of GIM-1-producing *A. pittii* in a German hospital due to a contamination of the pneumatic specimen transport system, but further investigation into the genetic platform of the MBL was not conducted. Kaase *et al.* have described a set of five different GIM-1 producing *A. pittii*.

With the rise of class B metallo- β -lactamases as a driving mechanism in resistance to carbapenems, it is of utmost importance to understand the genetic background of MBLs and ways of their transmission in *Acinetobacter*. As the amount of research into MBL-producing non-*baumannii* *Acinetobacter* has been little concerning European isolates and so far non-existent on isolates in Germany, it is of importance to gain insight into MBL-producing *Acinetobacter* isolates in Germany and to elucidate their genetic environment. The aim of our study was to identify the genetic platform of MBL-carrying *A. pittii* isolates from Germany and to characterize and analyse their resistome. Thus, 17 MBL-carrying *A. pittii* from different cities

in Germany, collected between 2010 and 2015 by the National Reference Center for Gram-negative hospital pathogens in Bochum, were examined. We hypothesized that the MBL-encoding gene would be plasmid borne in a substantial number of isolates and that these plasmids could bear similarities to one another. In contrast to so far existing studies, it was substantial to us, to not only focus on isolates encoding for one type of MBL, but to analyse and compare isolates producing different MBLs. We thus included four *bla*_{GIM-1}-carrying isolates from the study conducted by Kaase et al. in addition to another *bla*_{GIM-1}-carrying isolate, as well as isolates harbouring *bla*_{VIM-2}, *bla*_{VIM-4} and *bla*_{NDM-1}. Hence this is the first study examining a larger number of clinical *A. pittii* isolates in a European country as well as the first study worldwide to examine and compare the genetic platform of multiple different MBLs in *A. pittii*.

5.1. *A. pittii*, an emerging multi-drug resistant clinical pathogen in Germany

A surveillance study by Schleicher et al. conducted over 5 years (2005 – 2009) in Germany in 15 medical centres went on to show a higher prevalence of *A. pittii* over *A. baumannii*.²⁴⁸ Although this finding might not translate into a countrywide context, it underlines the importance of *A. pittii* in a clinical setting. As our study shows an array of MDR *A. pittii* isolated from clinical samples, we infer that *A. pittii* might be on its way to become an increasingly resistant pathogen in a clinical context. Newer studies published in recent years have found evidence of diverse clinically relevant *A. pittii* isolates with extensively drug-resistant phenotypes, which are equally mediated by resistance plasmids.²⁴⁹

Our study showed the presence of multiple MBL-carrying plasmids (encoding for either NDM-1, VIM-2 or GIM-1), which mediated resistance to carbapenems, the gold-standard for *Acinetobacter* treatment in Germany, alongside a variety of other resistance genes. Although resistance to cephalosporins and carbapenems can potentially be attributed to the chromosomally encoded *bla*_{ADC}-variant and *bla*_{OXA}-variant, respectively, resistance to these agents was demonstrated to be mediated or increased by the acquired MBL, by agar dilution resistance testing after conducting plasmid conjugation.

While resistance to sulfamethoxazole:trimethoprim (1:19) and tetracycline coincided with the presence of *sul1*- or *sul2*- and *tet*(39)-genes, respectively, there were a few inconsistencies regarding the alignment of genotypes and phenotypes for aminoglycoside resistance: The four isolates harbouring a chromosomally encoded *bla*_{NDM-1} were susceptible to all tested aminoglycosides which aligned with the absence of any aminoglycoside resistance-mediating genes. The other three isolates harbouring a plasmid-encoded *bla*_{NDM-1} showed resistance towards kanamycin and only intermediate susceptibility for amikacin, which was attributed to

the presence of the gene *aph(3')-VI* encoding for an aminoglycoside phosphotransferase. In the five GIM-1 producing isolates we additionally found elevated MICs for gentamicin, which was attributed to the presence of *ant(2'')-Ia* alongside *aph(3')-VI*, which encodes an aminoglycoside adenylyltransferase. In the isolates harbouring *bla_{VIM-2}* we could find high MIC values for all three aminoglycosides that have been tested. However, in isolate UKK-0548 we couldn't find an aminoglycoside modifying gene as a mediator of gentamicin resistance. Furthermore, the three isolates harbouring VIM-4 were resistant to gentamicin and kanamycin, but susceptible to amikacin, despite harbouring *aac(6')/Ib* which is described as a mediator for amikacin resistance.²⁵⁰ This could be due to an IS-element located upstream of this gene, which disrupts the promoter. However, as the isolates with chromosomally encoded MBLs were only subjected to short-read sequencing, longer chromosomal sequences were not at hand and a verification of this hypothesis was not conducted. Further investigation into aminoglycoside resistance mechanisms in the *Acinetobacter* genus might clarify these findings.

Fluoroquinolone resistance in our isolates was associated with mutations in *gyrA*- and *parC*-genes as this is also the main resistance mechanism known for *A. baumannii*.^{93,94,251,252} In all five ciprofloxacin-resistant isolates (with MICs ranging from 8 µg/ml to 64 µg/ml) a Ser81-Leu substitution was found in GyrA. UKK-0554, which showed a MIC of 16 µg/ml, had an additional Ser84-Leu substitution in ParC. Higher MICs of 64 µg/ml were associated with an additional Glu88-Lys substitution in ParC. GyrA and ParC substitutions were the same as those reported in *A. baumannii* but these were in the past numbered according to the corresponding mutations found in *E. coli* (Ser83-Leu in GyrA and Ser80-Leu and Glu84-Lys in ParC)^{93,94}.

Resistance genes were commonly found on plasmids in the isolates of our study. *bla_{GIM-1}* and *bla_{VIM-2}* were exclusively plasmid-borne, while resistance genes for sulphonamide resistance and aminoglycoside resistance were also mostly located on plasmid DNA. It appears that horizontal gene transfer could be a driving force in the acquisition of antimicrobial resistance in *Acinetobacter* in general, but particularly in *A. pittii*. As previously described conjugation in Gram-negative bacteria takes place via a T4SS. T4SS as mediators of horizontal gene transfer are widely distributed, but their genetic features in *Acinetobacter* have so far rarely been the topic of further research, although the presence of VirB-encoding genes has already been described in plasmids found in the *Acinetobacter* genus.^{194,253} All MBL-encoding plasmids examined in our study harboured only ten of the twelve genes encoding for the minimized VirB/VirD4 system. Genes encoding for VirB3 and VirB7 were not encoded on any plasmid, nor found encoded on other DNA segments from the isolates, be it chromosomal or plasmid DNA. Similar findings have also been shown in a recently published analysis on p-type T4SS in *Acinetobacter*.²⁴⁹ The lack of these two genes apparently does not affect the ability for conjugation, as all *bla_{NDM-1}*-harbouring plasmids and four of the five *bla_{GIM-1}*-harbouring

plasmids were able to conjugate. pGIM1_UKK-0265 was not conjugative as it was missing a gene section encoding for VirB6 as well as VirB8-10 but was otherwise completely identical to conjugative plasmid pGIM1_UKK-0555, which underlines the significance of the T4SS for conjugation in *Acinetobacter* spp. However, pVIM2_UKK-0548 and pVIM2_UKK-0547 were not conjugative despite harbouring the same set of 10 genes from the T4SS-complex. Except for the MBL-encoding region, *bla*_{VIM-2}-encoding and *bla*_{GIM-1}-encoding plasmids were almost identical and a promoter disruption through an IS-element could not be spotted as a culprit for the inability to exert conjugation. This finding indicates that there are other unknown determinants apart from the ten elements from the T4SS, that also play a role in conjugation, that are not present in the two isolates harbouring the *bla*_{VIM-2}-encoding plasmids. Further research could elucidate the structural requirements for a conjugation machinery in *Acinetobacter* and could identify indispensable genes for this process. Further understanding of the conjugation machinery in *Acinetobacter* might also deliver starting points for a combat against spread of MDR-conferring MGEs.

5.2. Plasmid analysis

In total, we were able to identify and consecutively examine 10 MBL-encoding plasmids. Although the plasmids differed in the encoded MBL, we found that the backbone of the plasmids – except for the MBL-encoding region – was mostly identical and only differed by diverse sets of insertion sequences. This indicates a relatedness between the different plasmids, although core genome multi locus sequence typing (cgMLST) analysis showed clinical unrelatedness between the plasmid-harboring isolates.

The first *bla*_{NDM-1}-carrying plasmids that were characterized in *Acinetobacter* spp. were pNDM-BJ01 and its close variant pNDM-BJ02, which is identical to pNDM-BJ01, except for a missing copy of insertion sequence IS_{Aba125}. Both plasmids were initially found in *Acinetobacter lwoffii* isolates from China¹⁹⁴. Interestingly enough, all NDM-harboring plasmids in our study were shown to be derivatives of pNDM-BJ01 (acc. no. JQ001791) or pNDM-BJ02 (acc. no. JQ060896), although we are not aware of any epidemiological link between our isolates and China. pNDM1_UKK-0538 showed 99.9 % identity to pNDM-BJ01, while pNDM1_UKK-0540 showed 99.9% identity to pNDM-BJ02. As with both pNDM-BJ01 and pNDM-BJ02, which do not harbour a known Rep protein⁸⁹, neither do the plasmids in our study. They are thus not eligible for plasmid typing through the AB-PBRT method.

The NDM-1-encoding plasmids included in our study showed significant similarity to a variety of NDM-1-encoding plasmids dispersed worldwide throughout the *Acinetobacter* genus, with coverages of 95% and above, as well as sequence identities of more than 99.9%.

Most of these findings are centered in Asia as well as in South America. In Asia most of these findings derived from China, e.g. pNDM-BJ01 and pNDM-BJ02. These similar plasmids were found in a variety of different *Acinetobacter* species.²⁵⁴⁻²⁵⁶ But there were single cases of NDM-harbouring plasmid-carriers in other Asian countries as well, such as India²⁵⁷ or Taiwan. Findings of South American isolates were equally frequent in Brazil²⁵⁸ or Chile²⁵⁹. Only few isolates harbouring close relatives of this plasmid were found in North America²⁶⁰. Plasmid pNDM-JVAP01 (acc. no. KM923969.1) was detected in a Turkish *A. dijkshoorniae* isolate²⁶¹ and is so far the only highly similar plasmid from the Middle East in the BLAST database. To our knowledge, there have thus far not been any findings of similar plasmids in central Europe, Africa or Australia and Oceania. It is unclear if scarce research is the reason for the absence of reports of similar plasmids, especially in Australia/Oceania and Africa or if there is an epidemiological link explaining the dispersal of similar plasmids in Asia (particularly China), the Americas (particularly South America) and through this study, too, in Germany/Europe.

All highly similar NDM-1-encoding plasmids were found in isolates from various *Acinetobacter* species and rarely in other species. This suggests that, despite the fact that various *Acinetobacter* plasmids were found to be hosted by different bacterial species, pNDM-BJ01-derivatives appear to have a rather narrow range in terms of their ability to replicate in different species.²⁶² Only three times has a plasmid with more than 40 % query coverage been described in species other than those of the *Acinetobacter* genus. Plasmid p3SP-NDM (acc. no. NZ_KP900015) was found in a Chinese *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) isolate,²⁶³ plasmid p06-1619-NDM (acc. no. KX832928) in a *Providencia rettgeri* isolate from Columbia, which shares 74% of the plasmid's sequence at an identity of 100.00 %.²⁶⁴ It shares most of the T4SS-encoding region as well as the entire resistance gene-encoding region. Furthermore, p_dmeb_c388_NDM1 (acc. no. CP095673), a plasmid found in an *Enterobacter hormaechei* isolate from Bangladesh showed an identity of 99.85 % with a query coverage of 47%. The highly similar regions included the resistance gene encoding regions, as well as *traA*, *traC*, *traD*, *trpF* and *GroES* and *GroEL* genes. While conjugation into other *Acinetobacter* species was demonstrated and is also underlined by the fact that highly identical plasmids were found predominantly in a wide variety of different *Acinetobacter* species, conjugation into *E. coli* has been shown to be unsuccessful in our research. Why plasmids with a similar genomic backbone were found in three other not closely related species, is unclear. However, the high sequence identities suggest some sort of relatedness. Further research could elucidate if conjugation into other not closely related species such as *Providencia* or *Enterobacter* spp. is possible. If not, other means of horizontal gene transfer could be involved in this inter-species transmission of gene sequences, such as transformation or transduction.

While the presence of *bla*_{NDM-1} is linked to a composite transposon, the plasmids harbouring *bla*_{VIM-2} or *bla*_{GIM-1} harbour the same integrase, Int11-like, which was not present in the plasmids harbouring *bla*_{NDM-1}. We therefore hypothesize, that the integration of the MBL-encoding genes as well as the other resistance genes into the respective plasmids through integration of gene cassettes is executed by the integrase. The fact that the majority of the resistance genes are grouped around the integrase supports this hypothesis. The integrase seems to derive from *TnAs3* or a related transposon, as an analysis with ISfinder²⁶⁵ showed high similarities between the integrase-encoding gene and *TnAs3*. BLAST-analysis conducted throughout this research revealed that the *bla*_{VIM-2}- or *bla*_{GIM-1}-harbouring plasmids from our study had not been described before. Only recently, plasmid p1OC061 (acc. no. CP087301) was found in an *A. baumannii* isolate from Münster, Germany, which showed significant similarity to the *bla*_{GIM-1}-encoding plasmids from our study. It showed a query coverage of 100.0% with sequence identities of 99.98%. It only differed in additional insertion sequences *ISAb44*, *ISAb1*, *ISAb19* and *ISAb14*. This means that, so far, all descriptions of derivatives of this integrase-harbouring plasmid-variant encoding either *bla*_{GIM-1} or *bla*_{VIM-2} stem from German isolates. Interestingly enough the integron found in pVIM2_UKK-0547 and pVIM2_UKK-548 shows similarities to the integron found in plasmids pFA267 (MT771289.1), pFA648 (acc. no. MT771290.1) in *A. nosocomialis* and pFA319 (acc. no. MT771291.1) in *A. seifertii* from South Korea²⁶⁶. The integron in these plasmids also harbours an integrase-encoding gene, *bla*_{VIM-2}, *QacEΔ1*, *sul1* and the N-acetyltransferase encoding gene in the same order, but does not encode for *aac(6')-Ib*. Instead *aacA7* and *aadA1* could be found in all three plasmids. Also, in pFA267 and pFA648 the resistance gene *aph(3')-VI* could be found, which was also present in pVIM2_UKK-0547 and pVIM2_UKK-0548. However, the rest of the backbone between these plasmids and the plasmids examined in our study is completely diverse.

Concurring with our initial hypothesis, we found that all plasmids examined in our study were shown to be highly similar, despite being harboured by mainly clinically unrelated isolates as demonstrated by their different sequence types. As the new variants of this plasmid harbouring an integrase and thus having acquired other MBL-encoding genes so far have only been found in Germany, they might even have developed as a variant of the initially globally dispersed *bla*_{NDM-1}-harbouring plasmid in Germany itself. Further research into MBL-based carbapenem resistance in *Acinetobacter* species in other countries in Europe could shed light on this matter and on possible epidemiological links.

In summary, these data show new variants of a globally spread plasmid in *A. pittii* in Germany that have acquired other resistance genes, including different MBLs. The source of the original *bla*_{NDM-1}-encoding plasmid is unknown as there are reports of similar plasmids throughout the world in multiple *Acinetobacter* species. The first report of that plasmid, however, derived from

China and the high prevalence of similar plasmids that were isolated in China could hint at a possible origin, although we must consider, that the high intensity of research in China in that area might act as a confounder. We hypothesize that this plasmid was already widespread in Germany in 2015. The data presented here suggest that this plasmid might also be found in other *Acinetobacter* species in Germany. We therefore recommend that the analysis of plasmids should not only concentrate on a single species as mobile elements in *Acinetobacter* appear to be widely compatible in-between species of the same genus. Furthermore, we should recognize plasmid-based resistomes more independently from their host-bacteria, as closely related plasmids were shown to reside in unrelated bacterial specimen according to cgMLST. A newer study has also found *A. pittii* isolates to not display restrictions in their dispersal, where isolates from different locations were shown to be related, while isolates obtained from the same location have shown to be genetically unrelated.²⁶⁷ As a new study shows, classic cgMLST-typing does not appear to work favourably to examine epidemiological relations in *A. pittii*. A new dcgMLST (distributed core genome multilocus sequence typing) typing method has been established by the authors, which has been able to sort isolates into three clustering levels, which enabled better understanding of epidemiological links.²⁶⁸

The emergence of this promiscuous plasmid, but also of plasmid-based resistance in general thus poses a significant threat to healthcare and efficacy of antimicrobial treatment. As the increased resistance to a multitude of agents in the antimicrobial armamentarium in clinically relevant members of the *A. baumannii* group contrast the diminishing pace in new antimicrobial development, in the future limited treatment options may lead to a higher incidence of treatment failure resulting in increased mortality. However, the understanding of intercellular mobility as a cause for increasing antimicrobial resistance could also open new targets for anti-infective therapy and may trigger new approaches for infection control and antimicrobial stewardship.

Despite the insights gained from this study, certain limitations should be acknowledged. First, the representativeness of the analyzed isolates may be limited, as the sample set is small and does not necessarily reflect the full diversity of strains circulating in different settings or regions. In particular, the geographical focus of the study may introduce a selection bias, potentially restricting the generalizability of the findings to other populations. The retrospective nature of the study may introduce inherent biases well. Furthermore, the interpretation of observed discrepancies between genotype and phenotype remains constrained by the lack of functional testing.

While genomic data can provide valuable insight, the absence of experimental confirmation limits definitive conclusions regarding the biological relevance of specific genetic determinants.

These aspects should be considered when interpreting the results and highlight the need for future studies incorporating broader sampling strategies and functional analyses.

6. References

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Lawrence, R. A. A review of the medical benefits and contraindications to breastfeeding in the United States. Arlington (VA): National Center of Education in Maternal and Child Health. <http://www.ncemch.org/pubs/PDFs/breastfeedingTIB.pdf> (Zuletzt abgerufen am 12.11.2008)

7. Appendix

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8. Advance publication of results

Publication

- Wunderlich A, Xanthopoulou K, Wille J, Wohlfarth E, Gerson S, Kaase M, Seifert H, Higgins PG. Carbapenem resistance in *Acinetobacter pittii* isolates mediated by metallo- β -lactamases. J Antimicrob Chemother. 2023 Feb 1;78(2):488-496.

Conference contributions

- Wunderlich A, Xanthopoulou K, Ertel J, Gerson S, Seifert H, Higgins PG. Carbapenem resistance in *Acinetobacter pittii* mediated by metallo- β -lactamases. 2018. 28th European Congress of Clinical Microbiology and Infectious Diseases. Madrid, Spain.
- Wunderlich A, Xanthopoulou K, Wille J, Gerson S, Seifert H, Higgins PG. Genetic Background of carbapenem non-susceptibility in *Acinetobacter pittii* isolates mediated by metallo- β -lactamases. 2018. DZIF Annual Meeting. Heidelberg, Germany.
- Wunderlich A, Xanthopoulou K, Wille J, Gerson S, Seifert H, Higgins PG. Dissemination of GIM-1-encoding plasmids in clinical *Acinetobacter pittii* isolates from Germany. 2019. 29th European Congress of Clinical Microbiology and Infectious Diseases. Amsterdam, Netherlands.