Opportunistic *Acinetobacter baumannii* and *Acinetobacter* sp. Isolates in Rural and Urban Wastewater Treatment Plants and Raw Manure and Biogas Digestates

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Giessen, Hessen

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Quotes

“The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.”

Sir Alexander Fleming, 1945

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List of publications

Publication I

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Publication II

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Publication III

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Publication IV

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Publication V

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Abbreviations

ACB	*Acinetobacter calcoaceticus-Acinetobacter baumannii* complex
ANI	Average nucleotide indentity
BGPs	Biogas plants
CRAB	Carbapenem resistant *Acinetobacter baumannii*
DDH	DNA-DNA hybridisation
EDTA	Ethylene diamine tetraacetic acid
ESBL	Extended spectrum beta lactamase
ESKPAE	*E. faecium, S. aureus. K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter*
EtBr	Ethidium bromide
GIs	Genomic islands
IS elements	Insertion seqeuence elements
LPS	Lipopolysaccharides
MALDI-TOF-MS	Matrix assisted laser desorption ionisation time of flight mass spectrometry
MATE-type	Multidrug and toxic compound extursion type
MEGA	Molecular evolutionary genetics analysis
MGEs	Mobile genetic elements
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
NDM	New Delhi metallo betalactamase
OmpA	Outer membrane protein
OMVs	Outer membrane vesicles
OXA-type	Oxacillinase-type
PyrG	CTP synthase
RND-type	Resistance nodulation cell-division type
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WGS	Whole genome sequencing
WWTPs	Wastewater treatment plants

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Hypotheses

This study was based on following hypotheses:

Hypothesis I

Diverse *Acinetobacter* species, and particularly different *A*. *baumannii* strains are released from livestock and human impacted sources into the environment.

Hypothesis II

Discharge of hospital wastewater (both human and veterinarian clinics) strongly influence the antimicrobial resistance phenotypes of *Acinetobacter* and the presence of clinically relevant *A. baumannii* strains (MLST types) in wastewater treatment plant.

Hypothesis III

Isolates of genus *Acinetobacter* (obligate aerobic bacteria) which also consist of opportunistic pathogens present in the livestock manure and influent of wastewater treatment plants are eliminated by anaerobic digestion of manure in biogas plants and sludge treatment process in wastewater treatment plant.

Hypothesis IV

The quaternanry ammonium compound present in the environment influence the presence of diverse genomic islands and elements in *Acinetobacter bohemicus* strains QAC-21b and KCTC 42081.

Summary

Genus *Acinetobacter* is diverse, and multi-drug resistant (MDR) *Acinetobacter* linked infections are problematic. Studies hitherto performed in extra-clinical settings, such as biogas plants (BGPs) and wastewater treatment plants (WWTPs), lacked diversity and antimicrobial resistance (AMR) of *Acinetobacter*.

This thesis studied the diversity and AMR of *Acinetobacter* in livestock manure, BGP digestates, a rural WWTP, and two urban WWTPs (receiving hospital/veterinary sewage), including upstream/downstream from the river receiving effluent located in Germany. Partial 16S rRNA/*rpoB* genes-based study showed highest diversity (8/14, phylotypes) in urban WWTPs influent. *A. baumannii* were found in manure, digestate and all stages of rural WWTP (except river upstream). BGPs and rural WWTP had novel sequence-types (18/23, STs) susceptible to antimicrobials—indicating these niches consisted diverse *Acinetobacter.* In urban WWTPs, *Acinetobacter* were also isolated from secondary sludge and anaerobic post-digestor filtrate, and mostly showed MDR phenotypes against carbapenems, colistin and ciprofloxacin, suggesting hospital waste inflow into WWTPs influenced their AMR profile and clinical relevance. Isolation of *Acinetobacter* from digestate, and treated sludge/anaerobic post-digestor filtrate indicated their survival in anaerobic condition, which was supported by the presence of genes encoding AMP phosphotransferase and adenylate kinase linked to processing of polyphosphates for energy. Comparative genomics of *A. bohemicus* strains from pig manure (QAC-21b, this study) and textile dying pond (KCTC 42081, Abbas et al. 2014) showed higher genomic contents of transposons/insertion elements, and genomic islands suggesting their adaptation to these environments containing quaternary alkyl ammonium compounds (QAACs), compared to type strain ANC 3994^{T} (forest soil, Krizova et al. 2014).

Considering *Acinetobacter* genome plasticity, the likelihood of selection/spread of AMR if released into environment via manure (sludge) must not be neglected. Experiments with *Acinetobacters* from various sources will help understand this process. Plant colonization study can provide insights into potential interactions in the rhizosphere and phyllosphere, revealing routes of *Acinetobacter* transmission to humans. Taking “One-Health” approach, future *Acinetobacter* genome comparison from different sources might help understanding evolution and adaptation from extra-clinical to clinical settings. This could help development of intervention strategies to control AMR spread in non-clinical environments.

Zusammenfassung

Die Gattung *Acinetobacter* ist vielfältig und Infektionen durch multiresistente (MDR) *Acinetobacter* sind problematisch. Bisherige Studien in außerklinischen Umgebungen wie Biogasanlagen und Kläranlagen mangelten an Vielfalt und antimikrobieller Resistenz (AMR) von *Acinetobacter*.

Diese Dissertation untersuchte die Diversität und AMR von *Acinetobacter* aus Gülle und des Gärrestes, einer ländlichen Kläranlage (ohne Krankenhaus-/Veterinärabwässer) und zwei städtischen Kläranlagen (die Krankenhaus-/Veterinärabwässer empfingen), einschließlich Zufluss/Abfluss nach der Abwassereinleitung in einen Fluss in Deutschland. Eine teilweise auf den Genen 16S rRNA/rpoB basierende Studie zeigte die höchste Diversität (8/14 Phylotypen) im Einlauf der städtischen Kläranlagen. *A. baumannii* wurde in Gülle, Gärrestes und allen Stufen der ländlichen Kläranlage gefunden (außer flussaufwärts). In Biogas-Anlagen und der ländlichen Kläranlage wurden neue Sequenztypen (18/23, STs) gefunden, die empfindlich gegenüber Antimikrobiotika waren–was darauf hindeutet, dass diese Nischen eine vielfältige *Acinetobacter* aufweisen. In den städtischen Kläranlagen wurden *Acinetobacter* auch aus dem Sekundärschlamm und dem anaeroben Nachgärungsfiltrat isoliert und zeigten größtenteils MDR-Phänotypen gegen Carbapeneme, Colistin und Ciprofloxacin, was auf den Einfluss von Krankenhausabfällen auf ihr AMR-Profil und ihre klinische Bedeutung hinweist. Die Isolierung von *Acinetobacter* aus Gärrestes und behandeltem Schlamm/anaerobem Nachgärungsfiltrat deutete auf ihr Überleben unter anaeroben Bedingungen hin, was durch das Vorhandensein von Genen unterstützt wurde, die für AMP-Phosphotransferase und Adenylatkinase kodieren, die mit der Verarbeitung von Energie aus Phosphaten in Zusammenhang stehen. Vergleichende Genomik der *A. bohemicus*-Stämme aus Schweinemist (QAC-21b, diese Studie) und einem Teich mit textilfärbendem Abwasser (KCTC 42081, Abbas et al. 2014) zeigte einen höheren Gehalt an Transposons/Insertionselementen und genomischen Inseln, was auf eine Anpassung an diese Umgebungen mit quaternären Alkylammoniumverbindungen im Vergleich zum Typstamm ANC 3994^{T} (Waldboden, Krizova et al. 2014) hindeutet.

Angesichts der Genomplastizität von *Acinetobacter* darf die Möglichkeit der Auswahl und Verbreitung von AMR bei Freisetzung in die Umwelt durch Düngemittel (Schlamm) nicht vernachlässigt werden. Experimente mit *Acinetobacter* aus verschiedenen Quellen werden dazu beitragen, diesen Prozess zu verstehen. Die

Untersuchung der Besiedlung von Pflanzen kann Einblicke in potenzielle Interaktionen in der Rhizosphäre und Phyllosphäre liefern und die Übertragungswege von *Acinetobacter* auf den Menschen aufzeigen. Ein "One-Health"-Ansatz schlägt vor, zukünftige Vergleiche des Genoms von *Acinetobacter* aus verschiedenen Quellen könnten die Evolution und Anpassung von außerklinischen an klinische Umgebungen verdeutlichen. Dies könnte zur Entwicklung von Interventionsstrategien zur Kontrolle der Ausbreitung von AMR in nicht-klinischen Umgebungen beitragen.

Overview

Taxonomic status, ecology, clinical significance and comparative genomics of *Acinetobacter* species—their presence in livestock-related and anthropogenic-impacted areas such as biogas plants and rural/urban wastewater treatment systems.

A historical perspective of genus *Acinetobacter*

The genus *Acinetobacter* was initially conceived in 1954 (Brisou and Prévot 1954). This genus taxonomically has long history of debate and change. In the advent of 20th century, a Dutch microbiologist, “Martinus Willem Beijerinck”, isolated first strain of *Acinetobacter* from soil by enrichment cultivation on a calcium acetate-mineral medium, and was described as *Micrococcus calcoaceticus* (Beijerinck 1911). Similar bacteria were also described and inadequately defined for a long time and assigned into multiple genera and species, including *Diplococcus mucosus* (Lingelsheim 1908), *Alcaligenes haemolysans* (Henriksen 1973), *Mima polymorpha* (DeBord 1939), *Moraxella lwoffi* (Audureau 1940), *Herellea vaginicola* (DeBord 1942.), *Bacterium anitratum* (Schaub et al. 1948), *Moraxella lwoffi* var. *glucidolytica* (Piechaud et al. 1951), *Neisseria winogradskyi* (Lemoigne et al. 1952), *Achromobacter anitratus* (Brisou 1953), and *Achromobacter mucosus* (Mannheim et al. 1962). The current genus designation, *Acinetobacter* (Greek meaning, ‘akinetos’, i.e., motionless) was proposed four decades later in 1954 by Brisou and Prévot to differentiate it from the motile organisms within the genus *Achromobacter* (Brisou and Prévot 1954; Peleg et al. 2008). In 1957, Brisou published unique species named *Acinetobacter anitratum* (Brisou 1957). Eventually in 1968, the genus “*Acinetobacter*” designation was widely accepted after Baumann et al. (1968) published comprehensive study of more than 100 strains of the oxidase-negative *Moraxellas* (*the Mima-Herellea-Acinetobacter* group of bacteria), which concluded that *Acinetobacter* belonged to a single genus, and further sub-classification into different species based on phenotypical characteristics was impossible. Only single species, named *Acinetobacter calcoaceticus* was considered, of which *Acinetobacter anitratum* was regarded as a synonym (Baumann et al. 1968). These findings led to the formal acknowledgment of the genus *Acinetobacter* in 1971 by the subcommittee on the “Taxonomy of *Moraxella* and Allied Bacteria” (Lessel, 1971). In the 1974 edition of Bergey`s Manual of Systemic Bacteriology, the genus *Acinetobacter* was listed with the description of a single

species, *A. calcoaceticus*, and classified the genus *Acinetobacter* within the family *Neisseriaceae* (Lautrop 1974). A major change in complicated taxonomic history of this genus was achieved in 1986 when Bouvet and Grimont distinguished 12 hybridization groups (genospecies/genomic species) by DNA-DNA hybridization (DDH: S1 nuclease method) assay, some of which were given formal species names, including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, and *A. lwoffii* (Bouvet and Grimont, 1986; Peleg et al. 2008). Intriguingly, molecular taxonomic study (DNA-rRNA hybridization) performed by Rossau et al. (1991) resulted in emended classification of the genus *Acinetobacter* inside new family named *Moraxellaceae*.

Contemporary taxonomy of *Acinetobacter* species

Following substantial progress in taxonomy over the last few decades, the genus *Acinetobacter* as currently defined, comprises Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive and oxidase-negative bacteria. Thus, current taxonomical classification is given as; Domain: *Bacteria*, Phylum: *Proteobacteria*, Class: *Gammaproteobacteria*, Order: *Pseudomonadales*, Family: *Moraxellaceae*, Genus: *Acinetobacter*, with a DNA G + C content of 38% to 47% (Peleg et al. 2008; Juni et al. 2015; Whitman et al. 2015). Interestingly, four species of genus *Acinetobacter*, i.e., *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, were closely related, and difficult to differentiate from each other by phenotypic properties (Gerner-Smidt et al. 1991). These species also showed high similarity based on DDH (Tjernberg and Ursing 1989), therefore these species were designated as the *A. calcoaceticus-baumannii* complex (ACB-complex) (Gerner-Smidt et al. 1991; Gerner-Smidt 1992). Nemec et al. (2011) defined the taxonomic, phylogenetic and nomenclatural status of *Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU following comprehensive analysis of the intra/inter-species diversity of the species included in the ACB-complex from the significant numbers of strains, and considering the microbiological and clinical relevance of *Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU, the formal binomial names *A. pittii* sp. nov. and *A. nosocomialis* sp. nov. were proposed for these species.

There are several groups of closely related strains of *Acinetobacter* which share higher DDH value of above 70% similarity, and are not feasible to be differentiated based on phenotypic property, thereby designated as genomic species or genospecies (Peleg et al. 2008). The valid taxonomic descriptions of some of these strains have been published recently, for instance, *A. colistiniresistens* (formerly genomic sp. 13BJ/14TU; Nemec et al. 2017; Bouvet and Jeanjean 1989; Tjernberg and Ursing 1989), *A. courvalinii* (formerly genomic species 14BJ; Nemec et al. 2016; Bouvet and Jeanjean 1989), *A. dispersus* (formerly genomic species 17; Nemec et al. 2016; Bouvet and Jeanjean 1989), *A. guillouiae* (formerly genomic species 11; Nemec et al. 2010; Bouvet and Grimont 1986), *A. bereziniae* (formerly genomic species 10; Nemec et al. 2010; Bouvet and Grimont 1986), *A. lwoffii* (formerly genomic species 9; Bouvet and Grimont 1986; Nemec et al. 2019), *A. nosocomialis* (formerly genomic species 13TU; Nemec et al. 2011; Tjernberg and Ursing 1989), *A. pseudolwoffii* (formerly genomic sp. 8 and taxon 23; Bouvet and Grimont 1986; Nemec et al. 2019), *A. seifertii* (genomic speies 'Close to 13TU'; Nemec et al. 2015; Gerner-Smidt and Tjernberg 1993), and *A. variabilis* (formerly genomic species 15TU; Krizova et al. 2015; Tjernberg and Ursing 1989). Thus far, the genus *Acinetobacter* comprises 70 species with valid names (https://lpsn.dsmz.de/genus/acinetobacter; Accessed November 2021), multiple species without valid names and several genomic species (www.szu.cz/anemec/Classification.pdf) (Accessed November 2021).

Identification of *Acinetobacter* species

Acinetobacter species are strictly aerobic Gram-negative rods which are difficult to destain, and may therefore be misidentified as either Gram-negative or Gram-positive cocci (Peleg et al. 2008; Juni 2015). Colonies are mucoid, and are generally non-pigmented when the cells are encapsulated. Cells generally occur in pairs and in chains of variable length measuring 0.9–1.6 × 1.5–2.5 μm in size. Cells are able to grow on most complex media. Most strains of *Acinetobacter* can grow in defined media with a carbon and energy source, for instance, acetate or lactate, using ammonium or nitrate salts or common amino acids, as a supply of nitrogen. Most commonly, amino acids like aspartic acid or glutamic acid serve as the source of carbon, energy, and nitrogen in a defined mineral medium. (Juni 2015; Whitman et al. 2015). Notably, *Acinetobacter* species of human origin can grow well on solid media often used in clinical microbiology laboratories, for instance, sheep blood agar or

tryptic soy agar at an incubation temperature of 37°C (Peleg et al. 2008; Towner 2006). Human originated isolates occasionally show haemolysis of sheep blood erythrocytes. For instance, isolates related to species *A. haemolyticus*, *A. colistiniresistens*, *A. courvalinii* and *A. disperses*, and several other currently not well-defined genomic species, such as *Acinetobacter* genomic species 6, 15BJ and 16 may produce haemolysis on sheep blood agar—a characteristic that is absent among the ACB-complex isolates (Bouvet and Grimont 1986; Bouvet and Jeanjean 1989; Peleg et al. 2008; Nemec et al. 2016, 2017).

Bouvet and Grimont (1986) initially proposed phenotypic identification scheme based on 28 different phenotypic tests. Later, they refined the scheme and included growth at 37°C, 41°C, and 44°C; acid production from glucose; gelatin hydrolysis; and assimilation of 14 various carbon sources (Bouvet and Grimont 1987a, 1987b). Despite the subsequent simplified scheme proved to be useful for several *Acinetobacter* species, nevertheless the four closely related members of ACB-complex could not be distinguished precisely by this system (Gerner-Smidt et al. 1991). However, this is inappropriate from a clinical perspective, since the ACB-complex combines three of the most clinically relevant species (*A. baumannii*, *A. pittii* and *A. nosocomialis*) with an environmental species, *A. calcoaceticus* (Peleg et al. 2008). It is prominent that the performance of commercial systems for species identification that are used in diagnostic microbiology are also unsatisfactory, particularly in cases involving ACB-complex isolates (Dijkshoorn et al. 2007). Notably, simple phenotypic tests usually performed during routine diagnostics for identification of several bacterial genera to the species level are not feasible for precise identification of even the most common *Acinetobacter* species (Peleg et al. 2008).

The lack of specificity of phenotypic tests-based identification has forced the development of multiple genotypic/molecular methods (Towner 2006), some of them applied for identification of *Acinetobacter* species are listed in Table 1. Amplified fragment length polymorphism (AFLP) analysis (Janssen et al. 1997; Nemec et al. 2001), amplified ribosomal DNA (rDNA) restriction analysis (ARDRA; Vaneechoutte et al. 1995; Dijkshoorn et al. 1998) and pulse field gel electrophoresis (PFGE; Seifert and Gerner-Smidt 1995) are among well-validated genotypic methods for identification and typing of *Acinetobacter* species (Dijkshoorn et al. 2007). Besides, among the genotypic/molecular methods that have been validated for identification of

Acinetobacter species, DDH remains the gold standard (Bouvet and Grimont, 1986). Although, these methods have assisted identification and understanding the clinical significance of *Acinetobacter* species in the recent years, they are extremely labour intensive for routine diagnostic microbiology, and their availability for the time being is confined mostly to reference laboratories (Peleg et al. 2008).

Multilocus sequence typing (MLST) which has recently gained profound attention is the molecular technique for strain characterisation that shows difference in several protein-coding (housekeeping) genes (Maiden 2006). The partial sequences of genes represent allele fragments and variation in the nucleotides assign to unique allele of the respective gene. Allele fragments from multiple genes give rise to unique allele numbers that are consequently combined in a specific order to obtain allele profile that defines the sequence type (ST). Strains that represent identical allelic profiles refer to the same STs, whereas strains that only share some of the alleles are phylogenetically related, and therefore designated as sequence complexes (CC: clonal complexes) (Maiden 2006; Glaeser and Kämpfer, 2015). The main advantage of the system includes its successful integration into public databases for molecular typing and microbial genome diversity (PubMLST) as the international databases of clinically important species that host collection of open-access and transportable sequence data (https://pubmlst.org/; Wellcome Trust; Jolley et al. 2018). MLST, which involves assessment of neutral genetic variation accumulated within a population, is now considered the gold standard for population structure study and global epidemiological investigations (Rafei et al. 2019; Gaiarsa et al. 2019). Considering the *Acinetobacter* species, preferably due to clinical significance of *A. baumannii*, two MLST schemes [Pasteur ($MLST^{Pas}$) and Oxford ($MLST^{Ox}$) schemes] are developed, and both target seven protein-coding genes of which three are in common (Rafei et al. 2019). Recently, Gaiarsa et al. (2019) found novel issue with Oxford scheme particularly due to presence of a paralog of the gene coding glucose dehydrogenase B (*gdhB*) locus (often annotated as *gdhB2*) in the majority (533/730) of the ACB complex genomes located in a different genomic region. On multiple occasions, allele sequences of the paralog *gdhB2* resulting from this duplication were wrongly used to assign novel Oxford scheme sequence types (STs) which originally do not exist. Another issue of the Oxford scheme is the putative recombination in the loci of *gpi* (coding glucose-6-phosphate isomerase) and *gyrB* (coding DNA gyrase subunit B),

which mostly affect phylogenetic analyses (Hamouda et al. 2010). Therefore, based on the suggestion of Gaiarsa et al. (2019), in our study (Pulami et al. 2020, 2023a; **CHAPTER 2 and 3**) Pasteur MLST scheme was used for typing, combined population structures studies and identifying domestic and international clones—since the Pasteur scheme provides higher precision for discriminating strains among clonal groups and is less influenced by homologous recombination. With the advent of whole genome sequencing (WGS) era, WGS-based approach such as core-genome MLST (cgMLST) is expected to outperform traditional typing methods ($MLST^{Ox}$ and $MLST^{Pas}$) which consequently will provide an unprecedented level of resolution able to address local and global epidemiological scales, and help understanding the evolutionary trajectory of emerging successful clonal complexes and population diversity over extended time-frames (Rafei et al. 2019).

In the clinical diagnostics, PCR amplification of species–specific DNA regions (for instance, the serine oxacillinase type *bla*$_{\text{OXA-51-like}}$ gene intrinsic to *A. baumannii* species) can be a valuable tool for confirmatory identification of individual pathogenic species (Turton et al. 2006b). Moreover, mass spectrometry techniques such as PCR-electrospray ionisation mass spectrometry (PCR-ESI-MS) (Schuetz et al. 2012) and matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF-MS) (Espinal et al. 2012; Li et al. 2018a) have shown promising results. These techniques allow rapid identification of bacterial species within few minutes; however, they comprise expensive equipment, fixed commercial database and require an expertise to evaluate data—therefore sequencing of *rpoB* (RNA polymerase subunit β) and *bla*$_{\text{OXA-51-like}}$ genes are still recommended (Álvarez-Buylla et al. 2012; Šedo et al. 2018).

The 16S rRNA gene has been used to identify *Acinetobacter* in general (Wagner et al. 1994). In the phylogenetic tree based on the 16S rRNA gene, *A. baumannii* and *A. nosocomialis* (formerly genomic sp. 13TU), and *A. calcoaceticus* and *A. pittii* (formerly genomic sp. 3), are in different branches while they have 65% DNA–DNA (DDH) similarity (Dijkshoorn and Nemec 2008). The tree based on the 16S rRNA gene is not consistent with the phylogenetic trees derived from *gyrB* (Yamamoto et al. 1999) *rpoB* (La Scola et al. 2006) and DNA recombination protein (*recA*, Krawczyk et al. 2002) sequences. Thus, it is now considered that 16S rRNA gene sequence analysis may neither show the correct phylogeny of some *Acinetobacter*

species nor be a reliable method for their differentiation (Dijkshoorn and Nemec 2008; Wang et al. 2014; Rani et al. 2017). Reliable identification of *Acinetobacter* isolates at the species level requires at least sequence analysis of the *rpoB*, and/or the *gyrB* (Gundi et al. 2009; Nemec et al. 2009). Considering reliability and affordability sequence-based analysis and typing system, such as, *rpoB* and *gyrB* analyses, *bla*$_{\text{OXA-51-like}}$ typing and MLST (Diancourt et al. 2010) were extensively used in our study (Glaeser et al. 2020; Pulami et al. 2020, 2021, 2023a, 2023b; **CHAPTER 2, 3, 4 and 5)**.

For instant and reliable delineation of individual *Acinetobacter* species in realm of ecology, epidemiology and pathogenicity, focus must be towards application of considerable effort to develop new and user's friendly molecular techniques that facilitate quick and precise identification. Therefore, the obvious challenge is to develop and validate the universal system for species identification, a technique most likely to be based upon DNA sequence analysis—provided that such system will be suitable for routine diagnostics. Nevertheless, the genus *Acinetobacter* has wide diversity, and comprises cluster of highly similar species, therefore development and reliability of a single method for *Acinetobacter* identification remains skeptical (Bergogne-Bérézin 2008).

Table 1. Methods for genotypic/molecular identification of *Acinetobacter* species

Method	Molecular target	Application	Reference
DNA sequence analysis	16S rRNA gene	Phylogenetic analysis/ identification	Nemec et al. 2009
	RNA polymerase subunit β (*rpoB*)	Phylogenetic analysis/ identification	La Scola et al. 2006
	DNA gyrase subunit β (*gyrB*)	Phylogenetic analysis/ identification	Yamamoto et al. 1999
	Recombinase/DNA recombination protein (*recA*)	Phylogenetic analysis/ identification	Krawczyk et al. 2002
	Intergenic spacer region (16S - 23S spacer rRNA gene)	Phylogenetic analysis/ identification	Chang et al. 2005

Single gene-based typing	*bla*$_{OXA-51}$-like gene	Identification and typing	Zander et al. 2012; Pournaras et al. 2014; Rafei et al. 2014, 2015
CRISPR-based ST	CRISPR-cas arrrays	Identification and typing	Karah et al. 2015
DDH	Total DNA	Species assignment/ differentiation	Bouvet and Grimont, 1986.
MLST	Housekeeping genes	Assessing population diversity, typing (Epidemiology)	Bartual et al. 2005; Diancourt et al. 2010
Core genome MLST (cgMLST)	Core genome	Assessing population diversity, typing (Epidemiology)	Higgins et al. 2017; Cerezales et al. 2019; Venditti et al. 2019
Amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA)	16S rRNA gene	Identification, typing	Vaneechoutte et al. 1995; Dijkshoorn et al., 1998
Amplified fragment length polymorphism analysis (AFLP)	Whole genome	Identification, typing	Janssen et al. 1997; Nemec et al. 2001; Da Silva et al. 2007
Pulse field gel electrophoresis (PFGE)	Whole genome	Typing	Seifert and Gerner-Smidt, 1995
Cell envelope protein-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	Cell envelope proteins	Identification, typing	Dijkshoorn et al. 1990
Lipopolysaccharides (LPS) analysis	LPS	Identification, typing	Pantophlet et al., 2002

‘Identification’ corresponds to the identification to species while ‘typing’ means the identification at the strain level (Dijkshoorn and Nemec 2008).

Ecology of *Acinetobacter* species

Acinetobacter spp. are saphrophytes, which are found ubiquitously in the natural environment (Towner 2006; Doughari et al. 2011; Juni 2015). It was estimated *Acinetobacter* represented 10^5 mg^{-1} and 10^5 mL^{-1} cells in soil and water, respectively (Baumann 1968). *Acinetobacter* spp. are considered as representatives of the normal flora of the human skin, pharynx, respiratory tracts, rectum and urine (Villegas et al. 2003) and other clinical specimens (Savov et al. 2002). The carriage rates of these bacteria in healthy person were found to be 42.5%, and as high as 75% in hospitalized patients, thereby considered to be natural residents of human skin (Seifert et al. 1997).

Apart from human, *Acinetobacter* spp. are also isolated from birds (Wilharm et al. 2017), animals (Rafei et al. 2015), fish and shrimps (Guardabassi et al. 1999), and faeces of domesticated animals (Poppel et al. 2016; Rafei et al. 2015). *Acinetobacter* spp. together with other Gram-negative genera such as *Escherichia* and *Klebsiella* have been associated with food contamination and spoilage (Doughari et al. 2011). They have been linked in the spoilage of meat, eggs, poultry and fish during storage (Towner 2006). *Acinetobacter* spp. have been recovered from vegetables such as cauliflowers, cabbages, cucumbers, lettuce, peppers, mushrooms, radishes and carrots, and fruits like apple and melon (Berlau et al. 1999). *Acinetobacter* spp. are also widely distributed in habitats like hospital settings, veterinary clinics, hospital and urban wastes, raw sewage, wastewater treatment plant settings and soil (Towner 2006; Doughari et al. 2011).

Notably, different members of this genus are associated to diverse habitats. *A. calcoacetius*, one of the ACB complex members and considered as soil bacterium (Doughari et al. 2011; Choi et al. 2012; Rafei et al. 2015), is also reported in sewage water (Maravić et al. 2016; Din et al. 2021), vegetable (Berlau et al. 1999; Rafei et al. 2015), animals (Rafei et al. 2015) and human skin (Chu et al. 1999), and microflora of mosquito *Aedes albopictus* (Minard et al. 2013). Remaining ACB complex complex, such as *A. dijkshoorniae*, *A. lactucae*, *A. seifertii*, *A. nosocomialis*, *A. pittii* and *A. baumannii* are also found in diverse habitats. *A. dijkshoorniae* isolated from clinical samples linked to different geographical locations across the world (Cosgaya et al. 2016), is a later heterotypic synonym of *A. lactucae* obtained from lettuce (Rooney et al. 2016; Dunlap et al. 2018). *A. seifertii* isolated from human clinical specimens

(Nemec et al. 2015) is also reported in soil, water and cattle faeces *(*Furlan et al. 2019). *A. nosocomialis* commonly linked to clinical specimens (Nemec et al. 2011), human skin (Chu et al. 1999), and vegetable (Berlau et al. 1999). *A. pittii* is known to be associated with diverse habitats, such as human skin (Chu et al. 1999), clinical specimens (Nemec et al. 2011), animal and meat (Rafei et al. 2015), soil (Rafei et al. 2015), vegetables (Berlau et al. 1999; Rafei et al. 2015). *A. baumannii* considered as a most important ACB complex member, apart from hospital settings and clinical specimens (Dijkshoorn et al. 2007), has been isolated from waste water (Hrenovic et al. 2016), sludge (Higgins et al. 2018), livestock and cattle manure (**CHAPTER 2 and 3**), soil (Furlan et al. 2018), vegetables (Berlau et al. 1999; Karumathil et al. 2016; Carvalheira et al. 2017), animal (Rafei et al. 2015), foods of animal origin (McLellan et al. 2018), milk and milk-based products (Rafei et al. 2015; Cho et al. 2018; Wareth et al. 2021) and birds (Wilharm et al. 2017; Lopińska et al. 2020).

Acinetobacter species are generally known to utilize aromatic compounds in the nature. The genome of *A. baylyi* ADP1 revealed that the catabolic genes for aromatic compounds are located in five genomic loci (Barbe et al. 2004). Similarly, comparative genomics and genome wide analyses revealed that both environmental-origin strains (*A. calcoaceticus* PHEA-2 and *A. oleivorans* DR1) and pathogenic strains (*A. baumannii* ATCC 17978) have genomic loci linked to catabolic pathways for diverse aromatic compounds (Barbe et al. 2004; Jung et al. 2011). This suggests the role of *Acinetobacter* in nutrient cycling in natural environments (Jung and Park 2015). *Acinetobacter* is able to degrade hydrocarbons primarily with respect to alkanes of diverse chain lengths. *Acinetobacter* spp. are frequently found in various hydrocarbon contaminated sites, including mangrove sediments, soils, Antarctic marine sediments, and pristine environments, resembling their alkane biodegradation potential (Kuhn et al. 2009; Kang et al. 2011; Rocha et al. 2013).

Acinetobacter is also known to be involved in degradation of phenolic compounds. Microbial degradation of phenolic compounds deals with utilization of these compounds secreted by crop roots, and upon accumulation in the soil, phenolic compounds exhibit allelopathic stress and inhibit development of plant, thereby restraining sustainable crop production. For instance, *A. calcoaceticus* CSY-P13 isolated from the rhizosphere, is known to be associated with microbial degradation of phenolic compounds, such as, ferulic acid (FA) and p-hydroxybenzoic acid (PHBA)

accumulated in soil and rhizosphere of continuously cropped cucumber—thereby, degrading the level of phenolic compounds in soil, and mitigating the stress of FA and PHBA in cucumber by activating antioxidant enzymes and reducing reactive oxygen species levels, and also by changing rhizosphere bacterial community and inducing soil enzymes (Wu et al. 2018).

Livestock manure, BGP digestates, and WWTPs sludge and effluent as source of diverse *Acinetobacter* species into the natural environment, and their implications in the 'One-Health'

Considering the clinical relevance of *Acinetobacter* species, several studies related to surveillance of *Acinetobacter* species in extra clinical environment, such as animal manure, cattle manure storage tank and avian (including poultry) have been reported (Rafei et al. 2015; Fernando et al. 2016; Wilharm et al. 2017; Hrenovic et al. 2019)—the focus has been towards *A. baumannii* in these studies.

In our study, we isolated phylogenetically diverse groups of *Acinetobacter* spp. isolates from livestock and human population sources (**CHAPTER 2 and 3**). Considering livestock sources, manure (BGPs input sample) and anaerobic digestate (BGPs output sample) were collected from five German farms with small scale on farm biogas plants (BGP1, BGP2, BGP4, BGP5, and BGP6). The farms used cattle manure (BGP1, BGP2, BGP6), pig manure (BGP4, BGP5) or mixed manure (cattle, pig and chicken; BGP5) for the biogas production. Only one BGP (BGP2) used a thermophilic process temperature for biogas production, while all other BGPs were operated with mesophilic process temperature. Similarly, considering human population influenced sources, three WWTPs: one receiving only wastewater without hospital derived wastewater (WWTP1) and two wastewater treatments plants (WWTP2 and WWTP3) which derived wastewater from human and veterinary hospitals were sampled. For all three WWTPs, water samples were analysed from the upstream and downstream of river receiving the effluent inlet.

Acinetobacter isolates were assigned to phylotypes, which were defined by the formation of distinct clusters in the phylogenetic trees with pairwise 16S rRNA gene and *rpoB* sequence similarities of at least 98.4–100% and 95.8–100% among the isolates present in the respective cluster following phylogenetic analyses. Here, multiple *Acinetobacter* sp. isolates were cultured from WWTPs and BGPs. On the

basis of partial 16S rRNA and *rpoB* genes, cultured isolates belonged to *A. baumannii*, *A. pittii*, *A. lactucae*, *A. oleivorans*, *A. calcoaceticus*, *A. bereziniae*, *A. gandensis*, *A. gerneri*, *A. indicus*, *A. wuhouensis*, *A. guillouiae*, *A. beijerinckii*, *A. tandoii*, and *A. ursingii*, *Acinetobacter* sp. ANC 4050, *Acinetobacter* sp. ANC 4051, *Acinetobacter* sp. LUH 1470, *Acinetobacter* sp. ANC 4218 and *Acinetobacter* sp. ANC 4945, respectively, and represented 14 different phylotypes (**CHAPTER 2**). Majority (97/132: 73.07%) were isolates of ACB complex, which consisted 52 isolates of *A. baumannii* (phylotype A-6). *A. baumannii* isolates were detected in all sampling point of WWTP1 (influent, effluent, activated sludge, digested sludge, river water downstream) except in upstream of receiving river. Considering the WWTP2, *A. baumannii* isolates were isolated from influent sewer, inflow/outflow of primary clarifier, primary sludge and secondary sludge. Similarly, *A. baumannii* isolates were cultured from inflow/outflow of primary clarifier, primary sludge, secondary sludge and anaerobic post-digestor filtrate of WWTP3. Furthermore, these isolates were detected in samples from BGPs with mesophilic operation condition, which included both input manure and output digestate of BGP6, and only output digestate of BGP1. Considering the BGP with thermophilic operation condition, *A. baumannii* isolates were isolated from both input manure and output digestate of BGP2 (**CHAPTER 2**). Majority of non-baumannii ACB complex isolates (44/45; phylotypes A-7.1 and A-7.2) were cultivated from WWTP1, WWTP2 and WWTP3, however only single isolate was cultured from input manure of BGP6 (**CHAPTER 2**). In WWTP1, which lacked anaerobic secondary treatment process of the sludge, these isolates were isolated from all sampling points which included influent, activated sludge, dewatered sludge, effluent and water samples downstream of the receiving river, except from water samples in upstream of the discharge of effluent in receiving river. These isolates were detected in influent, inflow/outflow of primary clarifier, primary sludge, secondary sludge and anaerobic post-digestor filtrate of WWTP2 and WWTP3 (**CHAPTER 2**). Intriguingly, ACB complex (including *A. baumannii*) isolates were obtained from both influent and effluent of WWTP1, however they were not isolated from effluent samples of WWTP2 and WWTP3, where anaerobic secondary treatment process of sludge was applied. This indicated the advantage of anaerobic secondary treatment process in preventing the release of ACB complex isolates into the environment via effluent discharge, which was also suggested in previous study (Hrenovic et al. 2016).

Based on Pasteur MLST scheme 23 novel and 12 known STs were determined (**CHAPTER 2**). Detection of majority of novel STs (18/23) from BGPs and WWTP1 (without hospital waste) in this study indicated that non-clinical environment harboured huge diversity of *A. baumannii* isolates which were phylogenetically distinct from those circulating in the hospital/veterinary settings (Zander et al. 2012; Wareth et al. 2019 and 2020). These isolates were still susceptible to clinically relevant MER (Meropenem), IMP (Imepenem), COL (Colistin), CIP (Ciprofloxacin), CAZ (Ceftazidime), AMK (Amikacin) and PIP (Piperacillin) antibiotics—which agreed with previous findings of phylogenetically diverse and susceptible isolates in various non-hospital sources, such as avians (Wilharm et al. 2017), bovine (Klotz et al. 2019), milk powder (Cho et al. 2018), and meat and manure from cattle (Rafei et al. 2015). However, three STs (ST2, ST25 and ST79) grouped as CC2, CC25 and CC79, and considered as representatives of globally distributed IC2. IC7 and IC5 lineages were found in the influent sewer, post-anaerobic digestor filtrate and secondary sludge of WWTP2 and WWTP3 of this study. ST types of these international lineages are present worldwide causing outbreaks in hospital settings in the Europe and across the globe (Di Popolo et al. 2011; Karah et al. 2011; Chagas et al. 2014; Sahl et al. 2015; Molter et al. 2016; Da Silva et al. 2018; Hamidian and Nigro. 2019; Camargo et al. 2020). These STs are not limited to humans and clinical settings, for instance ST2 was also detected in diseased pets in Germany (Ewers et al. 2017) and from sheep in Pakistan (Linz et al. 2018), and WWTP receiving hospital waste in Croatia (Higgins et al. 2018). Similarly, ST25 was among the dominant clones found in animals admitted to veterinary hospital in Switzerland (Püntener-Simmen et al. 2019), and ST79 was reported in WWTP treating hospital waste (Higgins et al. 2018) and river located in urban area in Brazil (Turano et al. 2016). Here, *A. baumannii* isolates from WWTP2 and WWTP3 represented hospital/veterinary linked STs (ST2: IC2, ST25: IC7 and ST79: IC5), and they were identified as 3MRGN-Ab/4MRGN-Ab [MDR *A. baumannii* isolates were defined as MRGN-Ab (multi- resistant Gram-negative bacteria) with regard to antibiotic susceptibility test results against various antibiotic groups recommended by the “Commission for Hospital Hygiene and Infection Prevention (KRINKO) at the Robert Koch Institute”, Germany (Wendt et al. 2012; Kaase 2016)]. Indeed, isolation of STs exhibiting multi-resistances (3MRGN-Ab/4MRGN-Ab) and phylogenetic relatedness to clinical lineages (IC2 and IC7) only from WWTP2/WWTP3 suggested that hospital/veterinary settings represented their primary source, and

clinical wastewater inflow influenced the resistance profile of *Acinetobacter* present in wastewater. Moreover, presence of cultivable *A. baumannii* and *A. pittii* in output digestate of BGPs, and secondary sludge and anerobic post-digestor filtrate of WWTP2/WWTP3 of this study indicated the survival of these obligate aerobes during anaerobic digestion process at thermophilic/mesophilic BGPs and secondary WWTPs. Higgins et al. (2018) suggested anaerobic environment in WWTPs and BGPs as putative ecological niches important in the epidemiology of this bacteria. Since, treated sewage sludge from WWTPs is used as manure, the risks of spread of clinically relevant MRGN-Ab STs into the agricultural environment cannot be excluded taking "One Health" as contemporary issue—our findings suggest proper treatment of sewage sludge and biogas digestate generated in the WWTPs and BGPs

Clinical manifestation and antibiotic resistance in *Acinetobacter* species

Members of *Acinetobacter*, in particular the species belonging to ACB complex, which comprises opportunistic nosocomial pathogen, for instance, *A. baumannii* has been associated with ventilator-associated pneumonia, bloodstream infection, soft tissue infection, urinary tract infection, post-surgery and traumatic injury related infection across the globe. *A. baumannii* signifies a major public health crisis due to its ability to acquire or develop resistance to multiple antibiotics (Dijkshoorn et al. 2007; Peleg et al. 2008; Lee 2017; Nasr 2020). *A. calcoaceticus* has been associated with cutaneous necrosis in immunocompromised patient (Nonaka et al. 2014). *A. pittii* and *A. nosocomialis* have been linked to blood stream infection among hospitalized patients (Wisplinghoff et al. 2012). *A. baumannii*, *A. pittii*, *A. nosocomialis* and *A. calcoaceticus* were reportedly isolated from the hospitalized patients with community acquired or nosocomial infections in Germany from 2005 to 2009 (Schleicher et al. 2013). Antimicrobial resistance among clinically important *Acinetobacter* species is a great concern of public health (Wong et al. 2017).

Mechanisms conferring antimicrobial resistances in *Acinetobacter* commonly involve enzymatic degradation of antibiotics, modification or protection of the target, and decreased permeability due to efflux of the antibiotics by active efflux pumps (Peleg et al. 2008). Antimicrobial resistance among *Acinetobacter* is intrinsic or acquired via HGT. Intrinsic resistances are conferred mainly by chromosomally encoded β-lactamases, efflux pumps, and reduction in membrane permeability (Peleg et al. 2008; Lee et al. 2017). Acquired resistance involves HGT of resistance genes

via mobile genetic elements such as plasmids and transposons (Da Silva et al. 2016; Hamidian and Nigro 2019). *Acinetobacter* spp. are known to exhibit resistance to multiple classes of antibiotics, such as β-lactams, quinolones, tetracyclines, chroramphenicol, macrolides, aminoglycosides, and polymyxin (Doughari et al. 2011; Cai et al. 2012). Among *Acinetobacter* species, *A. baumannii* is considered important clinical pathogen (Dijkshoorn et al. 2007; Peleg et al. 2008), therefore it is classified as one of the members of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) which are commonly linked to infections by antibiotic resistant bacteria (Rice 2008). Considering the antibiotic resistance and clinical significance of *A. baumanni*, it is listed as the number 1 priority pathogen, which constitutes serious threat to modern medicine by World Health Organization (Willyard 2017).

Carbapenem antibiotics, for instances meropenem and imipenem belong to the β-lactam family, and used against most β-lactamase-producing bacteria, including those producing ESBL (extended spectrum β-lactamase) enzymes (Bush and Jacoby 2010). Carbapenem resistant *Acinetobacter baumannii* (CRAB) produces carbapenemases that can hydrolyze the carbapenems (β-lactam antibiotics), which are usually considered as 'last-line antibiotics' against resistant pathogens (Papp-Wallace et al. 2011; Kempf and Rolain 2012). To date, β-lactamases have been classified based on the molecular structure classification of the 'Ambler' (Ambler 1980) and the functional classification of "Bush-Jacobi-Medeiros" (Bush et al. 1995; Bush and Jacoby 2010). On the basis of molecular structure (conserved and distinguishing amino acid sequences and motifs) based approach or the Ambler's classification, β-lactamases are classified into four classes: class A, B, C and D, respectively. Classes A, C, and D include enzymes that require serine as an enzyme active center for hydrolysis, whereas class B β-lactamases are metalloenzymes that utilize zinc ion to facilitate β-lactam hydrolysis (Ambler 1980; Peleg et al. 2008; Sawa et al. 2020). Furthermore, in the functional classification, β-lactamases are classified as group 1, 2 and 3 depending on the degradation of β-lactam substrates and the effects of inhibitors (Bush et al. 1995; Bush and Jacoby 2010).

Inactivation of β-lactam by β-lactamase enzyme is most important antimicrobial resistance mechanism among *Acinetobacter* species. β-lactam or carbapenem

resistance in *A. baumannii* occurs via both intrinsic or acquired β-lactamases (Da Silva et al. 2016; Lee et al. 2017). *A. baumannii* produces intrinsic β-lactamases such as AmpC-type cephalosporinase (*bla*$_{AmpC}$, class C β-lactamases) and oxacillinase (*bla*$_{OXA-51}$-type, class D β-lactamases) expressed at low levels (Héritier et al. 2005a; Merkier et al. 2006; Da Silva et al. 2016). However, insertion of IS*Aba1* upstream of the *bla*$_{AmpC}$ enhances the expression, and confers resistance to 3rd generation cephalosporins, but not to carbapenems (Corvec et al. 2003; Segal et al. 2004; Héritier et al. 2006). Notably, *A. baumannii* isolates bearing the IS*Aba1*-*bla*$_{OXA-51}$-like gene, showed higher rates of resistance to carbapenems (imipenem and meropenem) (Turton et al. 2006a). Both, *bla*$_{AmpC}$ and *bla*$_{OXA-51}$-type genes were present in all *A. baumannii* isolates of our study, however based on PCR mapping and comparative genomics, IS elements upstream of these genes were not detected (**CHAPTER 2 and 3**). Acquired carbapenem resistance mostly occurs via class D β-lactamases (oxacillinases type or *bla*$_{OXA}$-like, e.g., *bla*$_{OXA-23}$-like) and class B metallo-β-lactamases (MBLs, e.g., New Delhi metallo-β-lactamases, *bla*$_{NDM}$-types), which are largely responsible for clinical outbreaks worldwide (Kempf and Rolain 2012; Zarrilli et al. 2013). Oxacillinase are a heterogeneous group of β-lactamases with higher potential in hydrolyzing oxacillin than benzylpenicillin (Bush 2013). To date, the acquired carbapenem-hydrolyzing class D oxacillinases described in *A. baumannii* can be designated into following groups: *bla*$_{OXA-23}$-like, *bla*$_{OXA-24/40}$-like, *bla*$_{OXA-58}$-like, *bla*$_{OXA-143}$-like and *bla*$_{OXA-235}$-like (Poirel et al. 2006, 2010; Lee et al. 2017).

Acquired carbapenem-hydrolyzing enzymes are the main cause of carbapenem resistance among *A. baumannii* strains (Hamidian and Nigro 2019). Oxacillinases in general induce weak hydrolysis of carbapenems, and are often poorly expressed, hence they are not able to led clinically relevant levels of resistance on their own (Héritier et al. 2005b). However, their expression is often enhanced by the insertion of an upstream IS element, which provides a strong promoter for enhanced expression, causing high resistance levels (Turton et al. 2006a; Corvec et al. 2007; Mugnier et al. 2009). Due to a broad substrate spectrum, MBLs are able to hydrolyze all β-lactam antibiotics including carbapenems, except monobactams (Jeon et al. 2015). So far, several types of MBLs are reported in *A. baumannii* which included: *bla*$_{NDM}$-type, *bla*$_{VIM}$-type, *bla*$_{IMP}$-type and *bla*$_{SIM}$-type (Jeon et al. 2015; Da Silva et al. 2016; Lee et al. 2017). Among these MBLs, *bla*$_{NDM}$-type has been reported worldwide

(Da Silva et al. 2016). Class A β-lactamases hydrolyze penicillins and cephalosporins more efficiently than carbapenems, and are not widely reported unlike class D and class B β-lactamases (Jeon et al. 2015). A number of class A β-lactamases, including *bla*$_{TEM}$-type, *bla*$_{SHV}$-type, *bla*$_{GES}$-type, *bla*$_{CTX-M}$-type, *bla*$_{SCO}$-type, *bla*$_{PER}$-type, *bla*$_{VEB}$-type, *bla*$_{KPC}$-type, and *bla*$_{CARB}$-type, have been reported in *A. baumannii*. Some of these enzymes, for instances, *bla*$_{TEM-1}$, *bla*$_{CARB-4}$, and *bla*$_{SCO-1}$ are narrow-spectrum b-lactamases, while other enzymes (for e.g., *bla*$_{PER-1}$, *bla*$_{PER-2}$, *bla*$_{PER-7}$, *bla*$_{TEM-92}$, *bla*$_{CARB-10}$, *bla*$_{SHV-5}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-15}$ and *bla*$_{VEB-1}$) are ESBLs (Lee et al. 2017). Some class A β-lactamases are able to hydorlyze carbapenems, and considered as carbapenemases, such as *bla*$_{GES-14}$ and *bla*$_{KPC-2}$, have been reported in *A. baumannii* (Bogaerts et al. 2010; Martinez et al. 2016). β-lactamase genes (*bla*$_{KPC}$-type, *bla*$_{OXA-48}$, *bla*$_{VIM}$-type) were not detected in all *Acinetobacter* isolates obtained from BGPs and WWTPs (**CHAPTER 2 and 3**), following screening by multiplex PCR using primers and amplification conditions as described by Monteiro et al. (2012). However, acquired carbapenemase *bla*$_{OXA-23}$-like gene was detected in MDR *A. baumannii* isolates isolated from WWTP receiving hospital waste (**CHAPTER 2**) indicating hospital sewage as their source.

Genome wide analysis of resistance genes, phage-like regions, resistance islands, genomic islands, QAACs and heavy metal tolerance efflux pumps genes in *Acinetobacter* species

Recent advancement in whole-genome sequencing (WGS) technologies and bioinformatic tools have enhanced the study of bacterial pathogens with unprecedented resolution, enabling phylogenomic studies and screening of genetic contents of outbreak associated lineages, and comparison of these lineages with given strains of interest (Popovich et al. 2017; Schürch et al. 2017; Quainoo et al. 2017). Currently, there exists 5766 *A. baumannii* genomes publicly available in the GenBank non-redundant and WGS databases (https://www.ncbi.nlm.nih.gov/genome/?term=Acinetobacter+baumannii) (Accessed on November 2021). The first *A. baumannii* genome was sequenced in 2006, strain ATCC 17978 (Smith et al. 2007), followed by an epidemic global clone (GC) one (GC1 or ST1) strain AYE in France, a non-clonal strain SDF associated with human body lice (Fournier et al. 2006), and a multidrug-resistant GC2 (ST2) strain ACICU recovered from Hospital in Italy (Iacono et al. 2008). In between 2008–2010, genomes

of some additional strains, including three CRAB GC1(ST1) strains (strain AB0057, AB056 and AB059) isolated from hospitalized military personnel were also sequenced in the USA (Adams et al. 2008, 2010). The accessibility and affordability of the short-read sequencing technologies rendered exponential increment in the number of sequenced genomes of *A: baumannii* from 2014 onwards (Hamidian and Nigro 2019). Few studies have compared genomes of *A. baumannii* strains originated from clinical and non-clinical environment (Vallenet et al. 2008; Yakkala et al. 2019).

In this study, we sequenced the whole genomes of six *A. baumannii* strains isolated from livestock manures (input materials) and digestates (output materials) of German biogas plants (**CHAPTER 3**). Additionally, one strain of *A. bohemicus* strain QAC-21b was also genome sequenced, which was isolated from pig manure from German pig farm (**CHAPTER 4**). The sequencing of whole genome shotgun libraries was done applying the dual index paired-end (v3, 2 × 300 bp) approach for the Illumina MiSeq platform as recommended by the manufacturer (Illumina, San Diego, USA). The NCBI Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) was used for annotation of the genome (Tatusova et al. 2016). Comparative genome analyses were performed in EDGAR 3.0 (Blom et al. 2016; Dieckmann et al. 2021). Genomes of *A. baumannii* and *A. bohemicus* strains have been deposited in NCBI under bioproject 'PRJEB35515' and 'PRJNA224116', respectively. Genomes of *A. baumannii* and *A. bohemicus* strains were compared to *A. baumannii* ATCC 19606^T (NZ_ACQB00000000) and *A. bohemicus* ANC 3994^T (NZ_APOH00000000). The details of comparative genomics and genome wide analyses are provided below (**CHAPTER 3** and **4**).

The genomes of *A. baumannii* strains carried multiple efflux pump-related genes (**CHAPTER 3**). The higher MIC values against oxacillin, the 3rd generation cephalosporin ceftiofur (with or without clavulanic acid), and against florfenicol, temocillin, fosfomycin and chloramphenicol (**CHAPTER 3**) could be due to the basal-level expression of RND (resistance-nodulation-cell division) type efflux genes like *adeABC* (regulated by two component regulatory operon, *adeR-adeS*) and *adeIJK*, multidrug and toxic compound extrusion (MATE) type efflux gene, e.g. multidrug efflux *abeM*, chloramphenicol resistance gene *craA* (reviewed by Coyne, Courvalin and Périchon 2011), fosfomycin resistance efflux gene *abaF* (Sharma et al. 2017) and *ampC* cephalosporinase (Corvec et al. 2003). All these genes were not located in

resistance islands but rather were intrinsic to the core genomes (**CHAPTER 3**). As expected, all *A. baumannii* strains in our study lacked point mutations in conserved regions of the AdeRS system that were reported by Yoon et al. (2013) mostly among multidrug-resistant clinical isolates of *A. baumannii*. Transposition of IS*Aba1* into the *adeS* was linked with overexpression of this efflux system in clinical isolates (Ruzin, Keeney and Bradford 2007); however, this gene was intact in all *A. baumannii* strains of our study (**CHAPTER 3**). Five out of six *A. baumanii* strains in our study lacked the *adeC*, this gene has been considered as non-essential for the multidrug-resistant phenotype conferred by the AdeABC efflux pump based on earlier studies (Marchand et al. 2004; Nemec et al. 2007). Insertion of IS element upstream was reported for hyperexpression of *ampC* in clinical *A. baumannii* (Corvec et al. 2003); however, all six strains lacked insertion at the upstream of this gene. Although *A. baumannii* is intrinsically resistant to these veterinary (florfenicol and ceftiofur) and clinical (temocillin, fosfomycin and chloramphenicol) antibiotics (Coyne, Courvalin and Périchon 2011), there are some reports on the use of fosfomycin in combination with other drugs, such as colistin, minocycline and polymyxin against *Acinetobacter* (Zhang et al. 2013; Sirijatuphat and Thamlikitkul 2014). In all six *A. baumannii* strains (isolated from livestock manure and digestates of German biogas plants), *comM* gene was intact and not interrupted by resistance island (**CHAPTER 3**), and our observation is in agreement with previous findings of disrupted *comM* gene by resistance island only among clinically related *A. baumannii* lineages (Hamidian and Hall 2018; Hamidian and Nigro 2019). The *comM* gene, which encodes putative ATPase linked to natural transformation among transformable species including nonpathogenic *A. baylyi* (Gwinn et al. 1998; Nero et al. 2018), is also considered hotspot for the integration of resistance island in antibiotic resistant clinical *A. baumannii* (Fournier et al. 2006; Bonnin et al. 2012; Hamidian and Hall 2018). These islands are structured mainly by mobile genetic elements (MGEs), such as transposons or integrons, and various resistance genes conferring multi-drug resistances (Hamidian and Hall 2018; Hamidian and Nigro 2019). First identification of AbaR type resistance island (AbaR1) in *Acinetobacter* was reported in *A. baumannii* strain AYE [lineage 1 of GC1 (ST1, European clone I)] which consisted an 86-kb large genomic region in which more than 40 antibiotic and heavy metal resistance genes were clustered, disrupting putative ATPase (*comM* gene) and creating unique target site duplications (TSDs, 5 bp length) during transposition (Fournier et al. 2006; Hamidian and Hall 2018). In carbapenem

resistant strain D36 belonging to lineage 2 of GC1 *A. baumannii*, resistant island AbaR4 (composite transposon Tn*2006* that carries the *bla*$_{OXA-23}$-like gene) was found in the same position in *comM* gene (Holt et al. 2016; Hamidian and Hall 2018). *A. baumannii* strains representing GC2 (ST2) are known to carry diverse AbGRI1-type GIs (genomic islands) with cluster of antibiotic and heavy metal resistance genes interrupting *comM* gene (Kim et al. 2017; Hamidian and Hall 2018). The acquisition of various GIs or resistance islands has been associated with evolution and contemporary rise of antibiotic resistant *A. baumannii* strains (Fournier et al. 2006; Post et al. 2010; Nigro et al. 2013; Holt et al. 2016; Hamidian and Hall 2018).

Similarly, we announced the genome sequence of *A. bohemicus* strain QAC-21b isolated in Mueller-Hinton (MH, Carl-Roth) agar containing benzyldimethyldodecylammonium chloride [BAC-C12, 50 mg L^{-1} as QAAC (quaternary alkyl ammonium compounds)] from a liquid manure of a conventional German pig farm. Whole genome shotgun sequencing and comparative genome analysis with *A. bohemnicus* type strain ANC 3994^{T} and ANC 5076=KCTC 42081 (formerly type strain of *A. pakistanensis*, Abbas et al. 2014; Nemec and Radolfova-Krizova et al. 2016) and other members of *Acinetobacter* revealed the genetic basis to QAAC tolerance. Comparative genomics showed the presence of genes coding for QAACs efflux pumps such as RND type efflux pump (*adeIJK*), MATE family efflux pump (*abeM*), SMR family ef-flux pumps (*abeS* and *sugE*) in the chromosome (**CHAPTER 4**), the combined action of these intrinsic efflux pumps might be responsible for reduced susceptibility to the QAACs. Previous studies had shown the association of *adeIJK* with reduced susceptibility to benzalkonium chloride (QAAC) in clinical *A. baumannii* (Damier-Piolle et al. 2008; Rajamohan et al. 2010; Lin et al. 2017). The *abeM* was known to be associated with efflux of QAAC in *Acinetobacter* (Su et al. 2005; Lin et al. 2017). Similarly, *abeS* was known to be associated with reduced susceptibility to benzalkonium chloride in clinically associated *A. baumannii* strain AC0037 (Srinivasan et al. 2009). The *sugE* was associated with reduced susceptibility to benzalkonium chloride in *Escherichia coli* (Chung et al. 2002; He et al. 2011). In *A. baumannii*, RND efflux pump AdeABC and its two-component system regulator AdeRS promoted the fitness in the presence of benzalkonium chloride (Knauf et al. 2018). Comparative genomic showed absence of this efflux system in strain QAC-21b and all other genomes of *Acinetobacter* species, except those

represented *A. baumannii* (**CHAPTER 4**), which proved the linkage of this system mostly among clinically associated *Acinetobacter* members (Marchand et al. 2004; Lin et al. 2017; Coyne et al. 2011; Yoon et al. 2013; Richmond et al. 2016). QAACs are common ingredients of pesticides, disinfectant and detergents in agriculture and animal husbandry (Mulder et al. 2018). As reported by German Society of Veterinary Medicine at least quarter of disinfectants used contained QAACs (DVG 2015). Bioavailable concentrations of QAACs can result cross-resistance (Singer et al. 2016) and co-selection of resistance to antibiotics and QAACs (Webber et al. 2015, Mulder et al. 2018). BacMet database showed the presence of copper resistance protein efflux genes (*pcoA, pcoB, copA, copB, copC* and *copD*), respectively in the genome (**CHAPTER 4**). These genes were previously reported among the copper tolerant strains of *A. baumannii* (Williams et al. 2016; Thummeepak et al. 2020).

Multiple intact phages were found to be integrated into the genome of strain QAC-21b. The intact phage-like regions in QAC-21b were similar (Phaster score: 140-150) to *Mannheimia* phage vB_MhM_3927AP2 (NC_028766.1), and *Acinetobacter* phage YMC11/11/R3177 (NC_041866.1). In Strain QAC-21b, two intact phage-like regions located in contigs NZ_CAJJDZ010000001 (phage region size: 37.5 kb) and NZ_CAJJDZ010000002 (phage region size: 32 kb) carried genes coding for integrase, transposase, terminase, tRNA, and phage portal, head, tail, plate, fibre and phage-like protein, including the *attL* and *attR* recognition sites, and multiple hypothetical proteins. Meanwhile, the phage region of *A. bohemicus* ANC 3994^{T} lacked genes coding for integrase, transposase and terminase, and *attL* and *attR* recognition sites were absent (**CHAPTER 4**), which are required for termination, integration and lysis for propagation inside the host bacterium (Casjens 2003; Canchaya et al. 2003; Labrie et al. 2010). Strain KCTC 42081 also possessed four incomplete phage-like regions in the genome (**CHAPTER** 3). The presence of several phage related genes integrated into the genomes of ANC 3994^{T}, strain QAC-21b and KCTC 42081 was in agreement with the previous findings of multiple phage-linked DNA regions in the genome of *Acinetobacter* species (Touchon et al. 2014). The presence of multiple genes encoding hypothetical proteins in all phage-like regions of strain QAC-21b might be associated with environmental (pig manure) adaptation. These phage-like regions help bacteria to gain antimicrobial resistance, adaptation across changing environments, and can provide novel virulence characteristics to the host bacterium

(Brüssow, Canchaya and Hardt 2004). Multiple studies had reported presence of phages in genome of members of the genus *Acinetobacter*, for instance, *A. baumannii* was considered as polylysogenic as it harbored multiple integrated phages in the genome (Snitkin et al. 2011, Touchon et al. 2014; Badawy et al. 2020; Loh et al. 2020).

Most notably, putative GIs of *A. bohemicus* ANC 3994^{T} mostly contained genes coding hypothetical proteins, and harbored only single gene encoding transposase (IS605 OrfB family; locus tag: F994_02674). However, putative GIs of strain QAC-21b and KCTC 42081 carried genes encoding transposases, integrase, heavy metal efflux pumps (for e.g., *czcA, czcD, czcO, cusR, cusS, arsH, copA, copB* and *pcoA*), antimicrobial resistance (for e.g., *mdtB* and *mdtC*), virulence-associated protein and hypothetical proteins (**CHAPTER 4**). Microbial GIs are cluster of genes involved in the genome evolution and environmental adaptation. GIs are linked with symbiosis, metabolism, fitness, antimicrobial resistance and pathogenicity (Juhas et al. 2009). The mobile genetic elements (for e.g., transposon, IS elements, plasmids and pathogenicity island) are associated with contemporary rise of antibiotic resistance in *Acinetobacter* species, particularly those representing ACB complex (Peleg et al. 2008; Lean and Yeo, 2017).

Virulence of *Acinetobacter* species

Modern advances in approaches linked to genomic, phenotypic and infection model studies have assisted in determination of pathogenicity or virulence factors of *A. baumannii* (Antunes et al. 2014). According to Smith et al. (2007), *A. baumannii* is known to share considerable parts of its genome for pathogenicity by harboring about 16 identified gene islands implicated in virulence. It is now accepted that *A. baumannii* possess a considerable pathogenicity which enable bacterium to move and survive in different environment, form biofilm, capture micronutrients, secrete proteins, interact with host cells and evade host immune systems (Weber et al. 2017; Harding et al. 2018; Li et al. 2018b; Sarshar et al. 2021). Comparative genomics and analyses in virulence factor database (VFDB; Chen et al. 2005; http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) showed the presence of genes that code for most virulence factors like type II and VI secretion systems, outer membrane protein A (OmpA), putative polysaccharide export outer membrane protein (EpsA), iron starvation-related protein (Fe/S protein) and type I chaperone usher pili (Csu pili) in the chromosomes

of all six strains of *A. baumannii* isolated from German biogas plants (**CHAPTER 3**). Similarly, the genome of *A. bohemicus* strain QAC-21b showed multiple pathogenicity genes encoding OmpA, phospholipase D (PlcD) and two-component response regulator transcription factor (BmfRS) (**CHAPTER 4**). These virulence-associated molecular structures or proteins are required by *Acinetobacter* to thrive in both biotic and abiotic environments (Weber et al. 2017; Harding et al. 2018).

Survival of *Acinetobacter* in oxygen limited environment and their attachment to the sludge particles

Although, *Acinetobacter* is considered as obligate aerobes, its members have been known to survive in anaerobic or oxygen-limited environments such as anaerobic digesters (Supaphol et al. 2011; Baek et al. 2014; Jo et al. 2015) and activated anaerobic mesophilic sludge digester in wastewater treatment plants (Higgins et al. 2018). In lab scale tests, *A. baumannii* did not grow under anaerobic conditions, but survived an incubation period of four weeks under the same conditions (Higgins et al. 2018). The authors pointed out that anaerobic treatment also enables the survival and dissemination of this nosocomial obligate aerobic pathogen. Here, we confirmed these findings by isolation of phylogenetically diverse *Acinetobacter* spp., including ACB complex isolates in digestates of anaerobic BGPs and filtrate sample (post-anaerobic digestion of sludge) from rural/urban wastewater treatment plant (**CHAPTER 2 and 3**).

In agreement with findings of Higgins et al. (2018), all *Acinetobacter* isolates (including *A. baumannii* strains) from biogas plants (**CHAPTER 3**) were able to survive in anaerobic laboratory test conditions. As yet, it is still unclear how *Acinetobacter* can survive under anaerobic or oxygen limited conditions. Earlier studies have shown that *Acinetobacter* spp. were able to efficiently accumulate intracellular polyphosphates, and therefore contributing in a small extent to the phosphate elimination in sewage treatment plants (Fuhs and Chen 1975; Deinema et al. 1980, 1985; Wentzel et al. 1986, 1991; Stephenson 1987; Bark et al. 1992). Van Groenestijn et al. (1987) suggested that the accumulated polyphosphates in *Acinetobacter* cells act as a phosphorus reserve, and potentially be used as energy source by enzymatic processing of the polyphosphates through combined action of polyphosphates: AMP phosphotransferase and an adenylate kinase. Comparative genome analyses

revealed the presence of genes that code for these enzymes in all six strain of *A. baumannii* cultured from livestock manure and digestates of anaerobically operated German biogas plants (**CHAPTER 3**). The slurry tank in the pig farm from where the *A. bohemicus* strain QAC-21b was isolated represent oxygen limited environment. Comparative genomics showed the presence of genes encoding AMP phosphotransferase and adenylate kinase (**CHAPTER 4**) in the genome of this strain. The described enzymatic process can be an explanation for the survival of these aerobic organisms in anaerobic biogas plant or anaerobic sludge treatment in wastewater treatment plants, because the polyphosphate reserve in *Acinetobacter* cells can be vital under anaerobic environment conditions when these strict aerobic organisms have no other options to generate energy (Kortstee et al. 1994).

Considering the isolation of viable *Acinetobacter* isolates, including *A. baumannii* from the activated and digested sewage sludge, it has been suggested that *Acinetobacter* cells are attached into the flocs duing the sewage treatment process (Higgins et al. 2018). The attachment of *Acinetobacter* with the activated sludge particles was shown previously via in situ hybridisation techniques (Wagner et al. 1994). The authors showed that 3 and 7% of stained cells attached to activated flocs of aerobic and anaerobic treatment tanks represented *Acinetobacter*. Phuong et al. (2012) also performed in situ hybridisation using the probe of Wagner et al. (1994) and showed that 6.7 and 4% of stained cell in settled sludge and primary setting tank represented the *Acinetobacter*. The authors suggested the inter-genera coaggregation of flocs froming bacteria (e.g., *Pseudomonas* species) with non-flocculating *Acintobacter*. This intergeneric close association in the flocs and sewage particles might triggers the horizontal gene transfer leading to selection of bacteria against different antimicrobials and pollutants (QAACs). As revealed recently (Wolters et al. 2022), the anaerobically digested sewage sludge (biosolids) from German WWTPs harboured multiple pollutants such as antibiotics, heavy metals and desinfectants (QAACs), which can trigger HGT among closely associated bacteria (e.g., those present in sewage particels and flocs). This however must be proven through detailed experiments.

Conclusion

Detailed comparison of phylogenetic diversity and antibiotic resistance profiles of *Acinetobacter* sp. isolates (including those related to ACB complex) isolated from livestock manure (BGP input sample), digestate (BGP output sample) collected from five German farms with small scale on farm biogas plants (BGP1, 2, 4, 5, and 6), and three WWTPs [WWTP1: rural without known hospital/veterinary sewage, and WWTP2 and 3: both receiving urban, hospital and veterinary sewages], including upstream and downstream water of river receiving effluent was performed. Phylogenetic analyses (16S rRNA and *rpoB* gene based) showed 14 different phylotypes within the genus *Acinetobacter*, and the influent of WWTP2 represented highest diversity (8/14 phylotypes). ACB isolates represented the majority (95/130); *A. baumannii* (52/132) and *A. pittii* (40/132) were dominant phylotypes. *A. baumannii* was cultured from manure and digestate of BGPs, and all stages of WWTP1 (except river upstream). They were recovered from influent, inflow/outflow of primary clarifier, and primary/secondary sludge of WWTP2. Considering WWTP3, they were isolated only from inflow/outflow of primary clarifier and anaerobic post-digestor filtrate. Pasteur scheme MLST identified 23 novel and 12 known STs. Most novel STs (18/23) were isolated from BGPs and WWTP1; showed susceptibility to MER/IMP, COL, CIP, CAZ and PIP antibiotics—indicating livestock manure, digestate and rural WWTP without hospital waste inflow consisted diverse population of susceptible *A. baumannii.* Only, isolates from WWTP2 and 3 showed clinical linkage (ST2: IC2, ST25: IC7, ST79: IC5); represented multi-resistant Gram-negative *A. baumannii* (3/4MRGN-Ab) [resistant/intermediate to MER/IMP, CIP, CAZ and PIP] and additionally showed COL resistance. Isolation of MRGN-Ab (clinical STs) only from urban WWTP2 and 3 proved clinical settings as their primary source, and clinical wastewater inflow influenced antibiotic susceptibility profile among *Acinetobacter* isolates in WWTPs. Presence of cultivable *A. baumannii* in digestate (BGPs), and secondary sludge and anaerobic post-digestor filtrate (urban WWTP2 and 3) indicated their survivability in anaerobic condition at thermophilic/mesophilic BGPs and secondary WWTPs. The comparative genomic analyses showed the presence of genes coding for the AMP phosphotransferase and an adenylate kinase in the genome of *Acinetobacter* spp. isolates. The enzymatic processing of accumulated polyphosphates inside *Acinetobacter* cells by the combined action of these two enzymes can use

phosphorous as a source for energy—which could explain the survivability of these aerobic bacteria in oxygen limited or anaerobic condition applied in BGPs and WWTPs. Since, treated sewage sludge and digestate are used as manure; the risks of spread of clinically relevant MRGN-Ab into the environment cannot be excluded. Considering the genome plasticity of *A. baumannii*, the likelihood of selection of MDR phenotype via horizontal gene transfer (conjugation, transformation and transduction) and OMVs cannot be excluded—meanwhile exposure to humans via agriculture produce, water sources and animals might lead to colonization by MDR *Acinetobacter* strains. *In vitro* and *In vivo* assay-based determination of virulence of *Acinetobacter* sp. isolates were not performed, however, comparative analyses showed the presence of multiple virulence associated genes encoding pathogenicity related molecular structures and proteins, such as type VI and II secretion systems, type I chaperone usher pili, Acinetobactin, outer membrane protein A, two-components response regulator transcription factor, iron starvation-related protein, and polysaccharide export outer membrane protein in the genomes of *Acinetobacter* sp. isolates isolated from livestock manure, BGPs digestates and pig manure.

Prospectives

In prospective study, a detailed comparative study considering the behaviour of particle attachment and role in flocculation during sewage treatment for multiple *Acinetobacter* strains isolated from these environments will be performed to understand the selection of antimicrobial resistances in such niches and risk associated with their spread into the receiving environment, when treated sludge are applied as manure in the agriculture. In addition, plant colonization experiments with the ACB complex isolates cultured from this study will provide insights into putative interaction of these bacteria in rhizosphere and phyllosphere of the plant—which can also reveal the potential route of transmission and spread of potentially pathogenic *Acinetobacter* into the human populations. Also, considering the “One Health” as public issue, the prospective study considering genome-wide-analyses-based comparison of ACB complex isolates from livestock, manure, BGPs digestate, WWTPs (particularly the effluent and digested sludge), agricultural produce and the environment with clinical strains is suggested. This will help understanding the assumed evolution, and possibly identify the factors required for adaptation of this bacterium from extra-clinical settings towards clinics as various nosocomial lineages—that will be crucial for developing possible intervention strategies to reduce the spread of antibiotic resistances in the extra-clinical environments.

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CHAPTER II

High diversity of the emerging pathogen *Acinetobacter baumannii* and other *Acinetobacter* spp. in raw manure, biogas plant digestates, and rural and urban wastewater treatment plants with system specific antibiotic resistance profiles

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Contributions:

PK and SG were involved in organizing samples from German wastewater treatment plants and biogas plants in 2018, 2019 and 2020. DP and SG were responsible for planning all experiments. SG was responsible for supervising all experiments performed by DP. DP performed laboratory experiments. DP and SG analysed the data and wrote the manuscript. PK provided the laboratory facilities, and contributed to the final version of manuscript. PK reviewed and edited the manuscript. This project was funded by the Federal Ministry of Education and Research (BMBF)-funded JPI-EC-AMR JTC 2017 project ARMIS (Antimicrobial Resistance Manure Intervention Strategies, 01KI1733; given to PK) and the DFG Project 458460392 (given to SG). DP received the PhD scholarship from Justus Liebig University Giessen.

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High diversity of the emerging pathogen *Acinetobacter baumannii* and other *Acinetobacter* spp. in raw manure, biogas plants digestates, and rural and urban wastewater treatment plants with system specific antimicrobial resistance profiles

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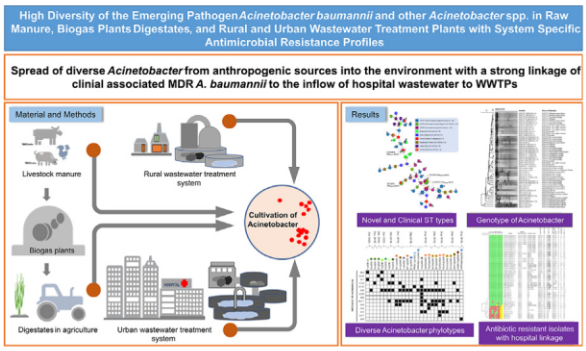
HIGHLIGHTS

- *High diversity of Acinetobacter* from raw and digested manure, rural and urban WWTPs
- Only *isolates* of urban WWTPs receiving clinical wastewater were multidrug resistant.
- *Acinetobacter* survives anaerobic wastewater treatments.
- *Acinetobacter* is more frequently detected in biosolids than effluent waters.
- Selective cultivation of *Acinetobacter* from the environment is problematic.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbapenem-resistant *Acinetobacter baumannii* causing immense treatment problems in hospitals. There is still a knowledge gap on the abundance and stability of acquired resistances and the diversity of resistant *Acinetobacter* in the environment. The aim of the study was to investigate the diversity and antimicrobial resistances of *Acinetobacter* spp. released from livestock and human wastewater into the environment. Raw and digested manure of small scale on farm biogas plants as well as untreated and treated wastewater and sewage sludge of rural and urban wastewater treatment plants (WWTPs) were studied comparatively. A total of 132 *Acinetobacter* isolates were phylogenetically identified (16S rRNA gene and *rpoB* sequence analyses) and 14 different phylotypes were detected. Fiftytwo isolates represented *A. baumannii* which were cultured from raw and digested manure of different biogas plants, and most stages of the rural WWTP (no hospital wastewater receiving) and the two studied urban WWTPs receiving veterinarian and human hospital wastewater. Multi-locus sequence typing (Pasteur_MLST) identified 23 novel and 12 known STs of *A. baumannii*. Most novel STs (18/23) were cultured from livestock samples and the rural WWTP. *A. baumannii* isolates from livestock and the rural WWTP were susceptible to carbapenems, colistin, ciprofloxacin, ceftazidime, and piperacillin. In contrast, *A. baumannii* isolates from the two urban WWTPs showed clinical linkage with respect to MLST and were multi-drug resistant (MDR). The presence of viable *A. baumannii* in digested manure and sewage sludge confirmed the survival of the strict aerobic bacteria during anoxic conditions. The study indicated the spread of diverse *Acinetobacter* from anthropogenic sources into the environment with a strong linkage of clinial associated MDR *A. baumannii* strains to the inflow of hospital wastewater to WWTPs. A more frequent detection of *Acinetobacter* in sewage sludge than effluent waters indicated that particle-attachment of *Acinetobacter* must be considered by the risk assessment of these bacteria.

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1. Introduction

The genus *Acinetobacter* is a member of the family Moraxellaceae within the Gammaproteobacteria. It comprises aerobic non-spore forming oxidase-negative Gram-stain negative bacteria which are found as free-living saprophytic bacteria ubiquitous in the environment (Kämpfer, 2014). Some species are clinically relevant, especially *Acinetobacter baumannii,* which accounts to the bacterial pathogens summarized as the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp.; Rice, 2008). Bacteria of this group are facultative pathogens which have acquired multi-resistances. Multi-drug resistance (MDR) strains cause immense problems in treatment of infections (Rice, 2008). Antibiotic resistant *A. baumannii* have been listed as one of the priority pathogens considering scope of resistance by the World Health Organisation (Willyard, 2017). *A. baumannii* belongs together with *A. nosocomialis*, *A. pittii*, *A. seifertii*, *A. calcoaceticus*, *A. djikshoorniae,* and *A. lactucae* to the *A. calcoaceticus–A. baumannii* (ACB) complex (Nemec et al., 2011, 2015; Cosgaya et al., 2016; Marí-Almirall et al., 2017). Species of the ACB complex cannot be reliably differentiated by phenotypic tests. Meanwhile, *A. djikshoorniae*, and *A. lactucae* are regarded as conspecific (Dunlap and Rooney, 2018). Although *A. baumannii* is responsible for most of the health care-associated nosocomial infection, other members of the genus, like *A. pittii*, *A. nosocomialis*, *A. radioresistans*, *A. lwoffii*, *A. ursingii*, and *A. calcoaceticus*, are increasingly reported as infecting agents (Wong et al., 2017; Schleicher et al., 2013). The expeditious emergence and dissemination of *Acinetobacter* had shown the successful adaptation of this bacterium to selective environmental pressures (Kempf and Rolain, 2012). *A. baumannii* is intrinsically resistant to several antibiotics which made carbapenems to referred antibiotic for treatment. As a consequence to the increased application of carbapenems for infection treatments the occurrence of carbapenem-resistance in *A. baumannii* is increasingly reported (Palavecino et al., 2022). Carbapenem-resistant *A. baumannii* strains produce enzymes that hydrolyze carbapenems (Perez et al., 2010; Papp-Wallace et al., 2011; Kempf and Rolain, 2012). Carbapenem-resistance in *A. baumannii* mostly occurs via class D OXA type serine oxacillinases (e.g., $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40/24-like}}$, $bla_{\text{OXA-58-like}}$) and class B metallo-β-lactamases (MBLs, e.g., $bla_{\text{NDM-1}}$), which are largely responsible for clinical outbreaks worldwide (Kempf and Rolain, 2012; Zarrilli et al., 2013). Most of those carbapenemase genes as $bla_{\text{OXA-23-like}}$ or $bla_{\text{OXA-}_{40}\text{/24-like}}$ are located on plasmids and are very critical for the spread of resistances among populations of *Acinetobacter* in clinical settings (Raible et al., 2017; Palavecino et al., 2022). Less is know about the spread among environmental *Acinetobacter* populations.

Livestock (manure) and human sources (wastewater) are considered as hotspots for the release of antibiotic resistant and potential pathogenic bacteria into the environment (Rizzo et al., 2013; Berendonk et al., 2015). Transmission routes into the environment can be raw or digested manure or sewage sludge used as fertilizers on agricultural fields, treated and/or untreated wastewater used for field irrigation, or effluent discharge of wastewater treatment plants (WWTPs) into adjacent freshwater systems (Heuer et al., 2011; Rahube and Yost, 2012; Rahube et al., 2014; Christou et al., 2017; Gupta et al., 2018; Wolters et al., 2022; Pérez-Valera et al., 2022). Biogas plants (BGPs) and WWTPs act as final interfaces between manure and wastewater and the receiving environment. Those treatment systems reduce the pathogen release, but do not completely eliminate it; antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) are steadily released into the environment (Berendonk et al., 2015). Viable ESKAPE pathogens and ARGs have been frequently detected in those systems (Dalkmann et al., 2012; Wolters et al., 2014, 2016, 2022; Schauss et al., 2015, 2016; Glaeser et al., 2016; Guo et al., 2017; Gupta et al., 2018; Müller et al., 2018; Tran et al., 2021; Wolters et al., 2022). The majority of the published studies on this topic have used molecular methods to determine ESKAPE pathogens and ARGs in total DNA extracts. Only few studies cultured *Acinetobacter* from raw and digested manure (Hrenovic et al., 2019; Schauss et al., 2015, 2016; Pulami et al., 2020), untreated and treated wastewater (Zhang et al., 2009; Higgins et al., 2018; Müller et al., 2018; Gupta et al., 2018), or sewage sludge (Higgins et al., 2018). Until recently, in Germany, *A. baumannii* was isolated from raw manure and digested manure of small scale on farm biogas plants (Schauss et al., 2015, 2016; Pulami et al., 2020), effluents of WWTPs and adjacent river systems (Müller et al., 2018), from different livestock animals and birds (Ewers et al., 2016, 2017; Wilharm et al., 2017; Klotz et al., 2019; Wareth et al., 2019), and livestock products (Cho et al., 2018; Wareth et al., 2020b). Moreover, *Acinetobacter* were detected by quantitative PCR (qPCR) together with other ESKAPE pathogens in the influents and effluents of German WWTPs (Hembach et al., 2017), sediment particles in effluent receiving rivers (Brown et al., 2019), and in the 16S rRNA gene amplicon datasets generated from total community DNA of digested sewage sludge (biosolids) from different German WWTPs (Wolters et al., 2022). Currently, there are no comparative studies available considering the diversity and resistance profiles of ACB and non-ACB *Acinetobacter* isolates from the different anthropogenic sources including livestock manure as well as wastewater treated in rural and urban WWTPs. The co-occurrence of several resistance causing pollutants, as antibiotics, biocides/disinfectant residues, and heavy metals strongly increasing the risk of the formation and spread of resistant bacteria, such as MDR *A. baumannii,* within the environment due to co-selection processes (Rizzo et al., 2013). Both, disinfectants, i.e. quaternary alkylammonium compounds (QAACs) and heavy metals can induce co-selection of antimicrobial resistances even at sublethal concentrations due to a broad range of co- and cross resistance mechanisms (Chapman, 2003; Maurya et al., 2020).

Our study is based on following hypotheses: (i) Different *Acinetobacter* spp. and more specifically different *A. baumannii* strains are released from livestock and human wastewater into the environment. (ii) Hospital wastewater (from human and veterinarian clinics) will strongly affect the resistance profiles of *Acinetobacter* and the presence of clinical relevant *A. baumannii* strains (sequence types, STs). (iii) Because *Acinetobacter* are considered as obligate aerobic bacteria, it is expected that anaerobic treatments of raw manure and sewage sludge have an impact on the release of *Acinetobacter* spp. into the environment.

To test these hypotheses *Acinetobacter* isolates were cultured from following sources: (i), livestock sources including raw pig and cattle manure and anaerobic digested manure obtained from small scale on farm biogas plants running with thermophilic or mesophilic process temperatures; (ii), human population derived wastewater including untreated wastewater, activated sludge, primary and secondary sludge, anaerobic digested sewage sludge, and effluent waters as well as water samples of effluent receiving rivers from a rural and two urban WWTPs. Two cultivation strategies were applied, a quantitative direct plating (DP) cultivation to count total *Acinetobacter* and a qualitative selective pre-enrichment (PE) cultivation to culture putative carbapenem-resistant *Acinetobacter*. The phylogenetic diversity of cultured *Acinetobacter* and the genetic diversity and antimicrobial resistance profiles of ACB isolates were studied in more detail.

2. Materials and methods

2.1. Livestock sources

Raw and digested manure were collected on five German farms with small scale on farm biogas plants (BGP1, BGP2, BGP4, BGP5, and BGP6). The farms used cattle manure (BGP1, BGP2, BGP6), pig manure (BGP4, BGP5 second sampling), or mixed manure (cattle, pig, and chicken; BGP5 first sampling) for biogas production. One biogas plant (BGP2) used a thermophilic process temperature, while all other biogas plants operated with mesophilic process temperatures. Further details on the studied biogas plants are provided as Supplementary materials (Table S1).

2.2. Human population sources

Three WWTPs were studied, one rural WWTP (WWTP1), which received only wastewater without hospital inflow, and two urban WWTPs (WWTP2 and WWTP3), which received wastewater from human and veterinary hospitals. The WWTPs contained a primary clarifier and a biological treatment with aerobic and anaerobic phases. The effluents of all WWTPs were discharged without additional disinfection after the secondary settling into rivers. Flowing surface water was collected from those rivers up and downstream of the effluent discharge points. Water samples were collected in the upper 30 cm of the water column from flowing water in a distiance of at least 1.5 m from the riverside. Further details to the studied WWTPs are provided as Supplementary materials (Table S1).

2.3. Samplings on biogas plants and WWTPs

Sampling campaigns on biogas plants were performed between July 2019 and April 2020. Some of the biogas plants were repeatedly sampled. At each sampling time point one sample was taken from the manure/slurry storage tank (representing the raw manure applied to the biogas plants) and one sample from the storage tank of the final digestates (digested manure). The rural WWTP was sampled in November 2019 and May 2020. At both time points activated sludge (AS), dewatered sewage sludge (S), and effluent (E) water were sampled. In May 2020, untreated wastewater (influent), water samples of the receiving river, upstream and downstream of the effluent discharge (RWup/down) were sampled in addition. The two urban WWTPs (WWTP2 and WWTP3) were sampled in parallel in August/September 2018. Following samples were taken: untreated wastewater collected from the sewer system (six samples, I), inflow and outflow samples of the primary clarifier (PCi and PCo), the effluent of the secondary clarifier (E), primary sludge (PS), secondary sludge (SS), anaerobically digested sewage sludge (ADS), and the filtrate of the post digester after centrifugation of the particulate fraction of the final digestates (anaerobic post digestor filtrate, APD-F), and river water samples collected upstream and downstream of the discharge point of the effluents (RWup/down). The overview of studied farm/ biogas plant and WWTP sampling points is provided as Supplementary material (Table S1). Samples were taken in 500 mL sterile PE bottles (Carl Roth, Germany), stored immediately at 6 °C and transported to the laboratory. All samples were processed within 24 h after collection for the cultivation dependent approach.

2.4. Cultivation of Acinetobacter on CHROMagar™Acinetobacter

For direct plating, bacterial cells were detached from 10 g of solid samples or 10 mL visible-particle containing sample material in autoclaved 250 mL SCHOTT bottles with 90 mL of 0.2 μm filter-sterilized tetrasodium pyrophosphate buffer (TSPP, 0.2 % *w/v*). The suspensions were incubated for 5 min on a horizontal shaker (120 rpm) in the dark. Larger particles were removed by sedimentation during subsequent incubation of the bottles for 30 min at RT in the dark. Cell suspensions from the upper layer of the bottles (30 mL; dilution 10^{-1}) were transferred to sterile 50 mL-plastic tubes (Greiner Bio-One GmbH, Germany) and used for analysis. Cell suspensions (10^{-1} dilutions) and liquid samples without visible particles were serially diluted (up to 10^{-3}) in autoclaved 0.9 (*w/v*)% sodium chloride (NaCl) solution. From each dilution, 100 μL were directly plated on CHROMagar™*Acinetobacter* (CA; CHROMagar, Paris, France) to count total cultured *Acinetobacter*. The agar media were partially supplemented with 5 mg L^{-1} cefsulodin (sodium salt hydrate, Sigma-Aldrich, Germany) to reduce growth of *Aeromonas* and *Pseudomonas* on the agar plates as recommended by the manufacturer. All plates were incubated under oxic conditions either for 24 h at 37 °C or for 4 h at 37 °C and subsequently at 44 °C overnight. Media and incubation conditions applied for individual samples are given in Table S2 (Supporting Information). The dry weight determination and the calculation of colony forming units (CFUs) per g dry weight or per mL liquid sample was carried out as described by Schauss et al. (2016). Colonies with a red colour were counted as putative *Acinetobacter* due to the instruction of the manufacturer.

A selective pre-enrichment method was applied to enhance the culturability of carbapenem-resistant *Acinetobacter*. Briefly, 0.1, 1, and 10 g of solid or visible particle containing samples were directly weighted into 0.9, 9, and 90 mL Lysogenic broth (LB; Sigma-Aldrich, Germany) containing 1 mg L^{-1} meropenem (MER, Cayman Chemical, Germany). Similarly, 1, 10, and 100 mL of liquid samples without visible particles were suspended in the same volume of double concentrated LB broth with a final concentration of 1 mg L^{-1} meropenem. After overnight incubation at 37 °C in the dark under continuous shaking at a horizontal shaker (180 rpm), subsamples (10 μL) were streaked on CA agar (Table S2). All plates were incubated overnight at 37 °C.

Following overnight incubation, if growth of presumptive *Acinetobacter* colonies (red colonies on CA) was obtained, up to 15 red colonies were selected per sample. The isolates were purified by singulation streaking on Mueller Hinton agar (MH, Carl Roth, Karlsruhe, Germany). For some of the samples an oxidase test was performed to preselect for oxidase negative colonies (cytochrome-c oxidase activity test, Bactident oxidase test strips, Merck). Fresh biomass of purified strains was suspended in newborn calf serum albumin (ThermoFisher Scientific, USA) and stored at −20 °C or − 80 °C for long-term preservation. A single loop of fresh biomass was suspended in 500 μL water (DNase and RNase free, PCR grade, ROTH, Germany) to generate cell lysates for subsequent molecular analyses by three cycles of freezing (−20 °C) and heating (1.5 min at 100 °C, heating block). Cell lysates were stored at −20 °C.

Following isolation of morphologically distinct colonies, the remaining total biomass on DP and PE agar plates was collected with an inoculation loop from agar plate in sterile 2 mL Eppendorf tubes containing 1 mL PCR grade water. Cell lysates were generated as described above.

2.5. Polymerase chain reaction (PCR) based identification of Acinetobacter isolates

Cell lysates obtained from pure cultures and total biomass from CA plates were used for PCR based detection of *Acinetobacter* by targeting an *Acinetobacter* specific conserved region of the 16S rRNA gene using primer system Ac436f (5′-TTTAAGCGAGGAGGAGG-3′) and Ac676r (5′-ATTCTA CCATCCTCTCCC-3′) which yielded in a 240 bp PCR product (Vanbroekhoven et al., 2004; Zhang et al., 2009). The PCR was performed in a total volume of 10 μL containing 1× PCR buffer, 0.2 mM dNTPs, 0.2 μM of each primer, 0.04 mg mL^{-1} bovine serum albumin (BSA), 0.02 U μL^{-1} Taq DNA polymerase (all chemicals expect primers from ThermoFisher Scientific, USA), and 1 μL cell lysate supernatant. The PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 32 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. A cell lysate generate from biomass of *A. baumannii* ATCC 19606^T was used as positive control. A cell lysate generated from biomass of *Pseudomonas libanensis* DSM 17149^T was used as negative control. PCR water was used instead of a DNA template as no-template control (NTC).

2.6. Genetic differentiation of isolates at the strain level by genomic DNA fingerprinting

All isolates were differentiated at the strain level by genomic DNA fingerprinting using BOX-PCR with primer BOXA1R (5′-CTACGGCAA GGCGACGCTGACG-3′; Versalovic et al., 1994). PCR amplification and agarose gel electrophoresis was performed according to Glaeser et al. (2013). Fingerprint patterns were analysed in BioNumerics version 8 (Applied Maths, Belgium) using the unweighted pair group method with arithmetic mean (UPGMA) clustering based on a pairwise distance matrix generated with the Pearson correlation coefficient. According to Franco et al. (2020) each unique fingerprint pattern was assigned as a distinct genotype.

2.7. Phylogenetic identification of bacterial isolates

Based on the strain differentiation by genomic DNA fingerprinting representatives of all different genotypes were phylogenetically identified by partial 16S rRNA gene sequencing as described previously (Glaeser et al., 2016). Pairwise 16S rRNA gene sequence similarities to closest related type strains were identified by BLAST analysis of the manually corrected 16S rRNA gene sequences against the curated EzBioCloud 16S rRNA gene sequence database containing all type strain 16S rRNA gene sequences with improved names (Yoon et al., 2017).

2.8. Phylogenetic placement of Acinetobacter isolates

The phylogenetic placement of the *Acinetobacter* isolates was performed based on partial 16S rRNA gene sequences and sequences of concatenated variable regions of the *rpoB* gene as described in detail by Pulami et al. (2020) using MEGA7 (Kumar et al., 2016). A ML tree based on *rpoB* sequences was calculated using the general time reversible model (GTR; Nei and Kumar, 2000) and 100 re-samplings. Pairwise sequence similarities for 16S rRNA and *rpoB* gene sequences were calculated by determining *p*-distances without using evolutionary models in MEGA7. Isolates were assigned to phylotypes, which were defined as distinct clusters in phylogenetic trees. Stains within a distinct cluster shared 98.65–100 % and 95.8–100 % pairwise 16S rRNA and *rpoB* gene sequence similarities, respectively. GenBank accession numbers of nucleotide sequences are MZ197905-MZ197972 and OL877388-OL897538 (16S rRNA gene) and OL956255-OL956524 (*rpoB* zone 1 and zone 2).

2.9. $bla_{OXA\text{-}51\text{-}like}$ and multi-locus sequence typing (MLST) of Acinetobacter baumannii isolates

Initial identification of putative *A. baumannii* isolate was performed by PCR amplification of a short fragment (353 bp) of the $bla_{\text{OXA-51-like}}$ gene using the primer system OXA-51-likeF (5′-TAATGCTTTGATCGGCCTTG-3′) and OXA-51-likeR (5'-TGGATTGCACTTCATCTTGG-3′) (Woodford et al., 2006; Feizabadi et al., 2008). PCR was performed in a total volume of 10 μL including 1 × PCR buffer, 0.2 mM dNTPs, 0.5 μM of each primer, 0.04 mg mL^{-1} BSA, 0.02 U μL^{-1} Taq DNA polymerase (all chemicals except primers from ThermoFisher Scientific, USA) and 1 μL cell lysate as template. Amplification conditions were as followed, initial denaturation at 95 °C for 3 min, followed by 32 cycles of 30 s at 95 °C, 30 s at 52 °C, 30 s at 72 °C, and a final extension at 72 °C for 10 min. PCR products were controlled on a 1 % agarose gel running for 45 min at 7.8 V/cm and stained with ethidium bromide. Positive and negative controls were identical to those used for the *Acinetobacter* 16S rRNA gene specific PCR. Isolates which were positive by the $bla_{\text{OXA-51-like}}$ gene screening were selected for $bla_{\text{OXA-51-like}}$ typing. Full length $bla_{\text{OXA-51-like}}$ genes were PCR amplified using primers OXA-69A (5′-CTAATAATTGATCTACTCAAG-3′) and OXA-69B (5′-CCAGTGGATGGATGGATAGATTATC-3′) (Héritier et al., 2005). PCRs and Sanger sequencing were performed according to Evans et al. (2008). Manually corrected gene sequences were used for BLAST analysis in NCBI BLAST to identify the $bla_{\text{OXA-51-like}}$ variants as described previously (Pulami et al., 2020). Comparison of the $bla_{\text{OXA-51-like}}$ sequences (both at nucleotide and amino acid levels) of isolates from this study to those represented by strains from different international clonal lineages (Zander et al., 2012) and sequence variants from Wilharm et al. (2017), Périchon et al. (2014), and Pulami et al. (2020), were calculated with the ML method. Phylogenetic trees were visualized using the interactive tree of the Life tree view tool iTOL version 5.7 (Letunic and Bork, 2007, 2021). All $bla_{\text{OXA-51-like}}$ positive isolates were additionally subjected to PCR mapping for localization of the insertion sequence element IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ genes according to previous studies (Segal et al., 2005; Turton et al., 2006), except the PCR was performed in a 10 μL volume. MLST analysis was performed with the Pasteur scheme (Diancourt et al., 2010) as described by Pulami et al. (2020) using the pubMLST database (https://pubmlst.org/organisms/acinetobacter-baumannii; Pasteur scheme; date of accession: 16-02-2021) for sequence type (ST) definition. A minimum spanning tree (MST) was calculated with phyloviz version 2.0 (http://www.phyloviz.net/goeburst/; Francisco et al., 2012; Nascimento et al., 2017) using the STs from this study, birds (Wilharm et al., 2017), WWTP with hospital sewage (Higgins et al., 2018), animals (Rafei et al., 2015; Klotz et al., 2019), and clinically-related STs from the pubMLST database. GenBank Acc. numbers of generated sequences are OL961390-OL961439 ($bla_{\text{OXA-51-like}}$ genes) and OL955912-OL956254 (MLST genes).

2.10. Seriation analyses

Seriation analyses were performed in PAST3 (https://folk.uio.no/ohammer/past/) to determine the occurrence patterns of different *Acinetobacter* phylotypes (different phylogenetic groups) in different samples. Analysis was performed according to Franco et al. (2020), based on an absence-presence (0/1) matrix using the algorithm described by Brower and Kile (1988). The analyses illustrated the presence of different phylotypes in various niches present in biogas plants, WWTPs, and receiving rivers.

2.11. PCR based detection of carbapenemase genes

Isolates were screened for the presence of carbapenemase genes using different multiplex-PCR systems. Screening for bla_{KPC}, $bla_{\text{OXA-48-like}}$, and bla_{VIM} was performed according to Monteiro et al. (2012). The presence of $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and $bla_{\text{OXA-58-like}}$ genes was determined according to Woodford et al. (2006) using 0.5 μM of each primer according to Feizabadi et al. (2008). PCR conditions were described above for $bla_{\text{OXA-51-like}}$ detection. Details to primer systems are provided in Table S3 (Supporting Information).

2.12. Antibiotic susceptibility testing of Acinetobacter isolates

Susceptibility of isolates to following antibiotics: temocillin, piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, ceftazidime/avibactam, ceftazidime/3-aminophenylboronic acid, ceftolozan/tazobactam, imipenem, meropenem, meropenem/ethylenediaminetetraacetic acid (metallo-β-lactamases inhibition), meropenem/3-aminophenylboronic acid (KPC inhibitor), amikacin, tigecycline, chloramphenicol, fosfomycin, trimethoprim/sulfamethoxazole, ciprofloxacin, levofloxacin, and colistin was investigated by broth microdilution using the MRGN MicronautS System (Merlin, Bornheim-Hersel) as described by Müller et al. (2018). Classification into sensitive (S), intermediate (I), and resistant (R) to tested antibiotics was done according to clinical breakpoints provided in CLSI (Clinical Laboratory Standards Institute; M100-ED30) (CLSI, 2020) and EUCAST (European Committee on Antimicrobial Susceptibility Testing; http://www.eucast.org/clinical_breakpoints/) databases. Based on the clinical breakpoints, minimum inhibitory concentration (MIC) values were automatically converted to SIR categories (Susceptible, Intermediate, Resistant) in BioNumerics v8. Categorical values [absent: 0, susceptible (S): 1, intermediate (I): 2, and resistant (R): 3] were used to construct a dendrogram based on hierarchical clustering using UPGMA in BioNumerics v8.

2.13. Copper and QAACs tolerance tests

Copper tolerance ($CuSO_4 \times 5H_2O$, Merck) of isolates was determined as described by Pulami et al. (2020). MIC values for the two tested QAACs, benzyldimethyldodecylammonium chloride (BAC-C12) and didecyldimethylammonium chloride (DADMAC-C10), were determined in broth microdilution assay following the CLSI guidelines (M100-ED30) as described by Heyde et al. (2020). Following concentration ranges were tested, 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 μg mL^{-1} for BAC-C12 and 0, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μg mL^{-1} for DADMAC-C10. Isolates were cultured on MH agar over night at 37 °C and suspended in 0.9 % sterile NaCl to the turbidity of a 0.5 McFarland standard. A subsample of 61 μL of the cell suspensions was used for the inoculation of

14 mL MH broth. Broth microdilution tests were performed in sterile 96 wells plate (Greiner Bio-one) in a total volume of 150 μL per test well containing 25 μL double concentrated MH broth, 25 μL water dissolved QAACs (six-fold concentration), and 100 μL of the bacterial suspension in MH broth. Plates was covered with sterile transparent plastic lids and incubated for 24 h at 37 °C in a humid chamber. The lowest concentration that suppressed growth was considered as MIC value for the respective QAACs.

3. Results

3.1. Enumeration and confirmation of Acinetobacter spp. cultured from livestock and human-derived wastewater sources

Putative *Acinetobacter* (red colonies on CA plates) were detected after direct plating on CA agar only in three out of 20 livestock samples and one out of nine samples of the rural WWTP. Positive growth of putative *Acinetobacter* was obtained only from one raw manure sample of BGP2 (BGP2-I, Apr. 2020) and one raw and digested manure sample of BGP6 (BGP6-I/-O, Oct. 2019). From the rural WWTP1 growth of putative *Acinetobacter* was only obtained from dewatered sewage sludge (WWTP1-S, Nov. 2019). In contrast, putative *Acinetobacter* were cultured from all 23 samples of the urban WWTPs (WWTP2 and 3) including water samples of the receiving river (Fig. 1A). The concentrations of putative *Acinetobacter* were in the range of 10^4–10^6 CFU g (dry weight)$^{-1}$ for raw and digested manure and the dewatered sewage sludge of WWTP1, and in the range of 10^2–10^5 CFU g (fresh weight)$^{-1}$ or mL^{-1} for WWTP2 and 3 samples (Fig. 1A). PCR based screening of biomass collected from red colonies confirmed the presence of cultured *Acinetobacter* for raw and digested manure samples and dewatered sewage sludge of WWTP1, all sewer samples, primary clarifier and primary sludge samples of WWTP2 and 3, as well as river water collected downstream of WWTP3. However, *Acinetobacter* were not cultured by direct plating from secondary sludge, digested sewage sludge, the post digestor filtrate, and effluent samples of WWTP2 and 3 (Fig. 1A).

3.2. Cultivation of putative carbapenem resistant Acinetobacter after selective pre-enrichment

Putative carbapenem resistant *Acinetobacter* (red colonies on CA plates grown after selective PE in the presence of meropenem) were detected in all livestock and WWTP samples, except the primary sludge of WWTP3. However, *Acinetobacter* growth was only confirmed for a subset of the samples by PCR screening of the biomass (Fig. 1B). The cultivation of putative carbapenem resistant *Acinetobacter* was confirmed for one time point for raw manure and for three time points for digested manure of BGP1, and for one time point for raw and digested manure of BGP2 and 6. From the rural WWTP1, putative carbapenem resistant *Acinetobacter* were detected by the first sampling date in activated sludge and dehydrated sewage sludge, and for the second sampling time in untreated wastewater, activated sludge, the effluent, and river water up and downstream of the effluent discharge, however not in dehydrated sewage sludge. In case of the urban WWTPs, *Acinetobacter* growth was confirmed in untreated wastewater, in influent and effluent samples of the primary clarifier, primary and secondary sludge and anaerobically digested sludge of WWTP2 and/or WWTP3 and the filtrate of the post digester of WWTP3, but not in the effluents of the urban WWTPs.

3.3. Phylogenetic diversity of Acinetobacter sp. isolates cultured from biogas plants and WWTPs

A total of 132 *Acinetobacter* isolates (Tables 1, S4, Supporting Information) were identified, of those 21 were cultured from livestock (13 from raw and eight from digested manure of BGP1, 2, and 6), 45 from the rural WWTP1, and 45 and 21 from the urban WWTP2 and WWTP3, respectively. Phylogenetic analyses based on partial 16S rRNA and *rpoB* gene sequences assigned

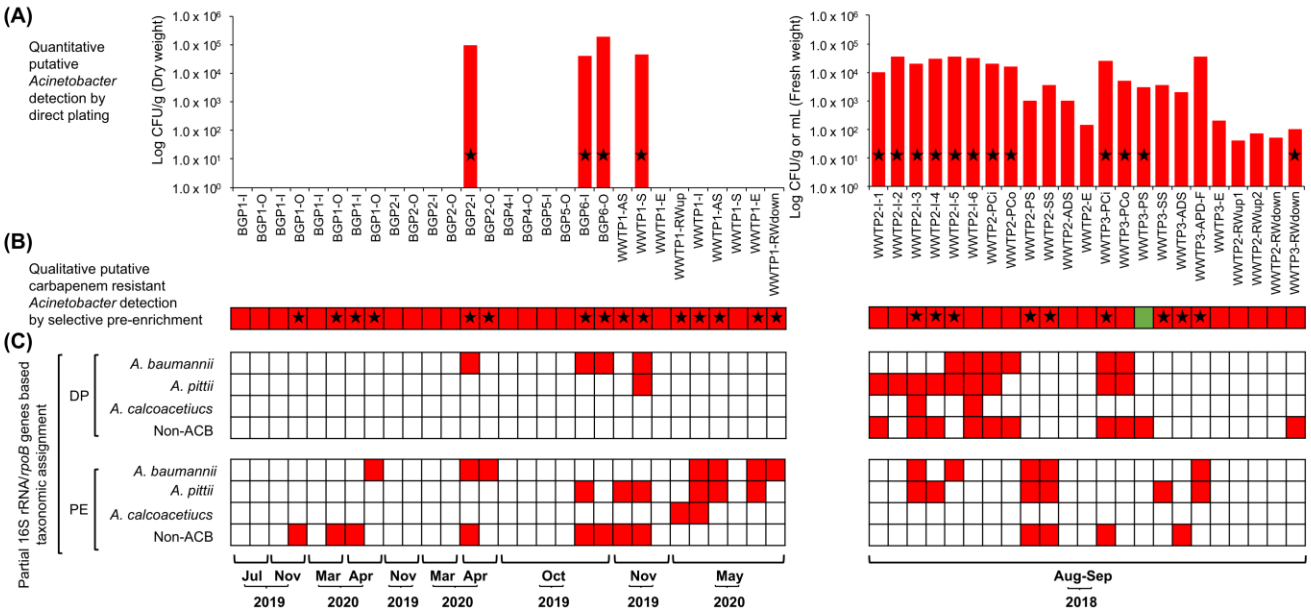


Fig. 1. Cultivation of *Acinetobacter* spp. from raw and digested manure (biogas plant digestates) and different sampling points of one rural (WWTP1) and two urban (WWTP2, 3) wastewater treatment systems. **(A)** Concentration of putative *Acinetobacter* cultured on CHROMagar™*Acinetobacter* (CA) by direct plating (DP). Total colony forming units (CFUs) of red colonies (putative *Acinetobacter*) were calculated per g (dry or fresh weight) or mL **(B)** Growth of putative *Acinetobacter* after selective preenrichment (PE) cultivation and streaking of pre-enrichment cultures on CA. Red boxes: positive growth; stars: confirmed detection of *Acinetobacter* by subsequent analyses. **(C)** Overview of the taxonomic assignment of *Acinetobacter* isolates cultured from CA media after DP and PE from the different studied samples. Red colored boxes indicated the phylogenetic identification (based on partial 16S rRNA and *rpoB* genes) of *Acinetobacter* isolates present in the respective samples. Identification was based on partial 16S rRNA gene sequence analysis (~920 nt for ACB complex isolates and ~ 600 nt for non-ACB isolates) with assignment at the genus level based on at least 98.7 % 16S rRNA gene sequence similarities to type strains of respective genera determined by BLAST analysis using the EzTaxon database, and supported by BLAST analysis of variable regions of *rpoB* gene (Nemec et al., 2009). Sample labels are described in text or Table S1.

Table 1
Overview of *Acinetobacter* sp. isolates cultured from the different samples investigated in this study. Phylogenetic assignment based on partial 16S rRNA and *rpoB* gene sequences. Phylotypes are assigned by next related *Acinetobacter* species. 16S rRNA gene and *rpoB* sequence similarities of the isolates and next related type strains of phylotype defining *Acinetobacter* species are given. Phylotypes were assigned according to the clustering in phylogenetic tress depicted in Fig. 2.

Pamhylotype	Phylogenetic assignment based on *rpoB* and partial 16S rRNA gene	Range of pairwise 16S rRNA gene sequence similarities	Range of pairwise *rpoB* sequence similarities	BGP 1				BGP4		BGP6		WWTP1				
				Input cattle manure (Apr.2020)	Digestate (Apr. 2020)	Digestate (Nov. 2019)	Digestate (Mar. 2020)	Raw cattle manure (Apr. 2020)	Digested manure (Apr. 2020)	Raw cattle manure (Oct. 2019)	Digested manure (Oct. 2019)	Activated sludge (Nov. 2019)	Dewatered sludge (Nov. 2019)	Inflow sewer influent (May 2020)	Activated sludge (May 2020)	Effluent (May 2020)
A-1	*A. gandensis* cluster	100 % (100 % to *A. gandensis*)	98.6–100 % (98.9–100 % to *A. gandensis*)	2				1								
A-2	*A.* sp. ANC 4218 cluster	(100 % to *A.* sp. ANC 4218)	(100 % to *A.* sp. ANC 4218)													
A-3	*A.* sp. ANC 4945 cluster	99.9 % (99.7–99.9 % to *A.* sp. ANC 4945)	97.6 % (97.5–98.9 % to *A.* sp. ANC 4945)	1				1								
A-4	*A. tandoii* cluster	99.9–100 % (98.4–98.5 % to *A. tandoii*)	97.6–100 % (97.6–100 % to *A. tandoii*)													
A-5	*A. beijerinckii* cluster	(100 % to *A. beijerinckii*)	(98.4 % to *A. beijerinckii*)													
A-6	*A. baumannii* cluster	99.5–100 % (99.7–100 % to *A. baumannii*)	98.9–100 % (98.9–99.9 % to *A. baumannii*)		1			3	1	2	2		4	5	11	5
A-7.1	*A. pittii*/*A. lactucae*/*A. dijkshoorniae*/*A.* sp. ANC 4050/*A.* sp. 4051/*A.* sp. KU 013TH cluster	99.2–100 % (99.2–100 % to members of non-*baumannii* ACB complex)	96.1–100 % (95.8–100 % to *A. pittii*/*A. lactucae*/*A. dijkshoorniae*/A. sp. ANC 4050/*A.* sp. 4051/*A.* sp. . KU 013TH)							1		1	3	5	1	2
A-7.2	*A. calcoaceticus*/*A.* sp. ANC 4221/*A.* sp. LUH 1470/"*A. oleivorans*" cluster	99.2–100 % (99.2–100 % to members of non-*baumannii* ACB complex)	97.1–100 % (96.7–99.9 % to *A. calcoaceticus*/*A.* sp. ANC 4221/*A.* sp. LUH 1470/"*A. oleivorans*")											2		

A-8	*A. ursingii* cluster	(100 % to *A. ursingii*)	(100 % to *A. ursingii*)						
A-9	*A. gerneri* cluster	99.2–100 % (99.5–100 % to *A. gerneri*)	98.9–100 % (99.4–99.7 % to *A. gerneri*)					1	1
A-10	*A. wuhouensis* cluster	(100 % to *A. wuhouensis*)	(98.6 % to *A. wuhouensis*)						
A-11	*A. guillouiae* cluster	(100 % to *A. guillouiae*)	(99.6 % to *A. guillouiae*)						
A-12	*A. bereziniae* cluster	99.9–100 % (99.9–100 % to *A. bereziniae*)	99–100 % (99.1–99.9 % to *A. bereziniae*)	1		2	1		2
A-13	*A. indicus* cluster	100 % (99.9 % to *A. indicus*)	99.4 % (97.3–97.5 % to *A. indicus*)		1		1		

WWTP1		WWTP2					WWTP3							
River upstream (May 2020)	River downstream (May 2020)	Inflow sewer influent (Aug. 2018)	Inflow of primary clarifier (Aug. 2018)	Outflow of primary clarifier (Aug. 2018)	Primary sludge (Sep. 2018)	Secondary sludge (Sep. 2018)	Inflow of primary clarifier (Aug. 2018)	Outflow of primary clarifier (Aug. 2018)	Primary sludge (Sep. 2018)	Secondary sludge (Sep. 2018)	Anaerobically digested sludge (Sep. 2018)	Anaerobic post-digestor filtrate (Sep. 2018)	River downstream (Aug. 2018)	**Total**
		1												**4**
				1										**1**
														2
		1	2				1	1	1					**6**
													1	**1**
	1	5 #(1)	2	2	1 #(1)	2	1	1				1		**52**
		10	3		1	3	3	1		4		2		**40**
1		2												**5**
		1												**1**
		1		1	1	1		1						**7**
		1												**1**
							1							**1**
						1					2			**9**
														2

Similarity values given in brackets represent sequence similarity values isolates shared with type strains/representing strains of cluster naming taxa. #Number inside the bracket represented the number of *Acinetobacter* isolate sharing identical DNA fingerprint pattern (BOX-PCR).

Fig. 2. Maximum likelihood trees based on **(A)** partial 16S rRNA gene sequences and **(B)** concatenated variable zones of the *rpoB* gene (Nemec et al., 2009) showing the phylogenetic relationship of *Acinetobacter* sp. isolates with members of genus *Acinetobacter*. GenBank accession numbers were given in parentheses. Bootstrap values >70 % (100 resamplings) were indicated. Bar: 0.01 substitutions per nucleotide position.

the isolates into 14 distinct phylotypes within the genus *Acinetobacter* (Table 1). The phylogenetic tree based on partial 16S rRNA gene sequences (Fig. 2A) showed 12 phylotypes that formed distinct clusters including type strains of described *Acinetobacter* species. The distinctness of the clusters was supported by high bootstrap values (70–100 %). Isolates of a non-*baumannii* ACB complex clade (A-7) could not be distinguished at the level of the 16S rRNA gene sequences (Fig. 2A), but by the *rpoB* gene based phylogenetic analysis (Fig. 2B).

Phylotypes A-3 and A-13 contained only isolates from livestock. Isolates from raw manure of BGP1 and 2 were assigned to phylotype A-3 and shared 99.7–99.9 % partial 16S rRNA gene sequence similarities among each other and with *Acinetobacter* sp. ANC 4945 (undescribed taxon). Isolates from digested manure of BGP1 and 6 represented phylotype A-13 (n = 2) and shared 99.9–100 % partial 16S rRNA gene sequence similarities among each other and with the type strain of *A. indicus*.

Several phylotypes were cultured only from WWTP samples. Phylotype A-9 isolates (n = 7) were cultured from all three WWTPs. Isolation sources were activated sludge and dewatered secondary sludge of WWTP1, sewer and outflow samples of primary clarifiers of WWTP2 and 3, and the primary and secondary sludge of WWTP2. The isolates shared 99.2–100 % and 99.5–100 % partial 16S rRNA gene sequence similarities among each other and with the type strain of *A. gerneri*. Six phylotypes, namely A-8, A-10, A-2, A-11, A-5, and A-4, were obtained only from the urban WWTPs. The first five phylotypes were only represented by single isolates from untreated wastewater (A-8, A-10) and the outflow of the primary clarifier (A-2) of WWTP2, and from inflow of primary clarifier (A-11) and river water downstream of the effluent discharge (A-5) of WWTP3. These isolates shared 100 % partial 16S rRNA gene sequence similarity with *Acinetobacter* sp. ANC 4218 and the type strains of *A. ursingii*, *A. wuhouensis*, *A. guillouiae*, and *A. beijerinckii*, respectively. Phylotype A-4 isolates (6 in total) were obtained from untreated wastewater (n = 1) and the inflow of the primary clarifier (n = 2) of WWTP2, and the inflow and outflow of the primary clarifier and primary sludge of WWTP3 (one isolate per sampling point). These isolates shared 99.9–100 % 16S rRNA gene similarities with each other and 98.4–98.5 % to the type strain of *A. tandoii* (Table 1 and Fig. 2A).

Isolates assigned to phylotypes A-1, A-12, and A-6 were cultured from both, livestock and WWTPs. Phylotype A-1 comprised isolates from raw manure of BGP1 (n = 2) and BGP2 (n = 1), and untreated wastewater of WWTP2 (n = 1). The phylotype shared 100 % 16S rRNA gene similarity with the type strain of *A. gandensis*. Nine isolates represented A-12, which were cultured from digested manure of BGP1 (n = 1), raw (n = 2) and digested manure (n = 1) of BGP6, dewatered sludge (n = 2) of WWTP1, secondary sludge (n = 1) of WWTP2 and anaerobically digested sludge (n = 2) of WWTP3. These isolates shared 99.9–100 % 16S rRNA gene sequences identity with each other and the type strain of *A. bereziniae*.

Phylotype A-6 comprised the highest number of isolates (52 out of 132). The isolates shared 99.5–100 % 16S rRNA gene sequence similarity among each other and 99.7–100 % with the type strain of *A. baumannii*. The A-6 isolates were cultured from three BGPs and all WWTPs. Isolates from biogas plants were obtained from raw manure of BGP2 and 6 (n = 3 and n = 2) and digested manure of BGP1, 2, and 6 (n = 1, n = 1, and n = 2). From WWTP1, A-6 isolates were cultured from untreated wastewater (n = 5), from activated sludge (n = 11), dewatered secondary sludge (n = 4), effluent (n = 5), and river water downstream of the effluent discharge (n = 1).

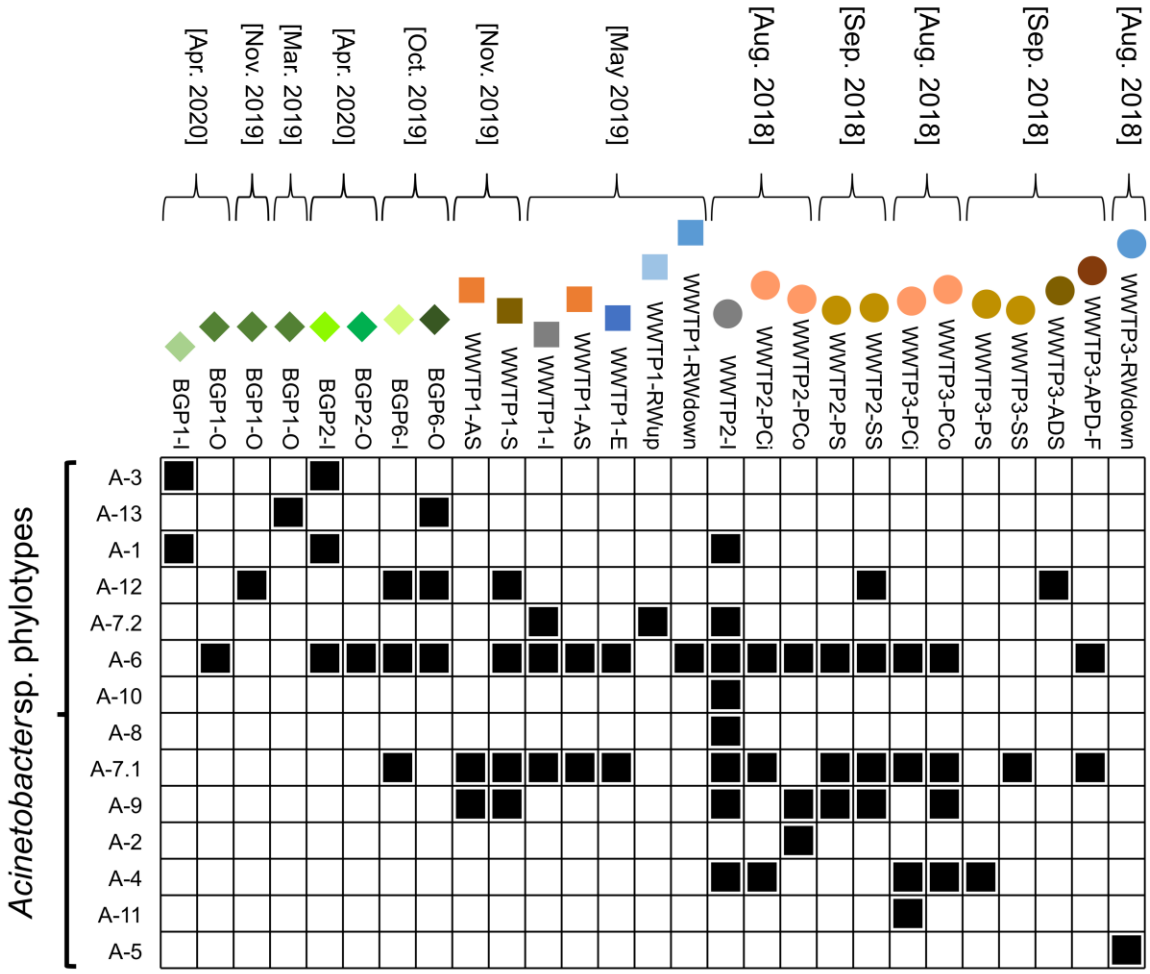


Fig. 3. Seriation analysis based on an absence-presence (0/1) matrix illustrating the presence of *Acinetobacter* phylotypes in different sampling points of biogas plants and wastewater treatment systems (WWTP1, without hospital wastewater inflow; WWTP2 and 3, receiving hospital wastewater).

Remaining A-6 isolates were cultured from untreated wastewater (n = 6), inflow (n = 2) and outflow (n = 2) of the primary clarifier, and the primary (n = 2) and secondary sludge (n = 2) of WWTP2, and inflow (n = 1) and outflow (n = 1) of primary clarifier, and anaerobic post-digestor filtrate (n = 1) of WWTP3.

The remaining 45 *Acinetobacter* isolates were assigned as a non-*baumannii* ACB complex clade in the 16S rRNA gene-based phylogeny. They shared 99.2–100 % partial 16S rRNA gene sequence similarities among each other and with type strains of *A. pittii*, *A. lactucae*, *A. dijkshoorniae*, *A. calcoaceticus,* and *"A. oleivorans"*, and other undescribed *Acinetobacter* species (*Acinetobacter* sp. ANC 4050 and *Acinetobacter* sp. LUH 1470) (Fig. 2A). The *rpoB* based phylogeny grouped the non-*baumannii* ACB isolates into two distinct clusters (Fig. 2B). Consequently, two additional phylotypes were defined. Phylotype A-7.1 contained 40 isolates which were cultured from raw manure of BGP6 (n = 1), untreated wastewater (n = 5), activated sludge (n = 2), dewatered sewage sludge (n = 3), and effluent water (n = 2) of WWTP1, from untreated wastewater (n = 10), inflow of the primary clarifier (n = 3), and primary (n = 1) and secondary (n = 3) sludge of WWTP2, and inflow (n = 3) and outflow (n = 1) of the primary clarifier, secondary sludge (n = 4) and the anaerobic post-digestor filtrate (n = 2) of WWTP3. The strains shared 95.8–100 % partial *rpoB* gene sequence similarity among each other and with type strains of *A. pittii*, *A. lactucae*, and *A. dijkshoorniae*, and with undescribed *Acinetobacter* sp. ANC 4050, ANC 4051, and KU 013TH. The remaining five isolates of the non-*baumannii* ACB complex clade formed phylotype A-7.2. The isolates were cultured from the river upstream (n = 1) of the effluent discharge and untreated wastewater of WWTP1 (n = 2) and untreated wastewater (n = 2) received from WWTP2. The strains clustered together with type strains of *A. calcoaceticus* and "*A. oleivorans*" and with *Acinetobacter* sp. ANC 4221 and LUH 1470 (Fig. 2B). The strains of phylotype A-7.2 shared 97.1–100 % *rpoB* sequence similarities among each other (Table 1).

Seriation analysis showed that the untreated wastewater samples from the urban WWTPs comprised the highest diversity of *Acinetobacter* phylotypes (8 out of 14). Following anaerobic treatment, the diversity decreased in the anaerobically digested sludge to one phylotype and two phylotypes in the anaerobic post-digestor filtrate compared to the untreated wastewater and the inflow of the primary clarifier (4 phylotypes) of WWTP3. Similarly, anaerobic treatment in BGP1 and BGP2 reduced the diversity of phylotypes in digestate (1 phylotype) compared with input manure (2 or 3 phylotypes) (Fig. 3). Cluster analyses based on BOX-PCR based genomic fingerprinting showed that most of the isolates represented unique genotypes (Figs. S1, S2, Supporting Information). In very few cases isolates asigned to the same phylotype shared identical fingerprint pattern indicating clonality (equal genotype).

3.4. Phylogenetic identification of non-target organisms (non-Acinetobacter isolates) in biogas plants and WWTPs

A selection of 190 non-*Acinetobacter* isolates that grew as red and non-red colonies on CA were also phylogenetically identified by partial 16S rRNA gene sequencing. Those isolates were assigned to 20 different genera (colors in brackets represent colony colorations on CA agar): *Pseudomonas* (red), *Citrobacter* (light-blue), *Empedobacter, Raoultella* (blue), *Chryseobacterium, Elizabethkingia* (blue), *Myroides* (red), *Stenotrophomonas* (light-red), *Aeromonas* (blue-red), *Comamonas* (light-black), *Lactobacillus* (brown), *Diaphorobacter* (brown), *Rhizobium* (black), *Pandoraea* (blue), *Achromobacter* (blue), *Burkholderia* (light-brown), *Bordetella* (light-brown), *Sphingobacterium* (light-black), *Brucella* (black), and *Cupriavidus* (light-black), respectively. *Pseudomonas*, *Myroides*, and *Empedobacter* were the genera representing most of the non-*Acinetobacter* isolates (Fig. S3; Table S5, Supporting Information).

3.5. Diversity of A. baumannii isolates based on OXA-51-like typing and MLST

The presence of a $bla_{\text{OXA-51-like}}$ genes in all phylotype A-6 isolates (n = 52) confirmed the assignment of the isolates to the species *A. baumannii*. Eighteen $bla_{\text{OXA-51-like}}$ gene allele variants were determined, OXA-51, −64, −65, −66, −69, −78, −91, −104, −106, −117, −120, −121, −375, −385, −391, −431, −688, and −862. Based on full length $bla_{\text{OXA-51-like}}$ gene sequences the *A. baumannii* isolates clustered in the phylogenetic tree with clinical, avian, and biogas plant isolates published in other studies (Fig. 4). The detected variants OXA-69, −66, −51, −65, and −64 are known to be associated with clinical *A. baumannii* lineages IC-1, −2, −4, −5, and −7, respectively. However, at the nucleotide level the OXA-51 and -65 variants detected among isolates from raw and digested manure of BGP2 (each, n = 2), untreated wastewater and effluent of WWTP1 (each, n = 2) and secondary sludge of WWTP2 (n = 1) were not identical to that of clinical lineages IC-4 and IC-5 (Fig. 4). One isolate of the digested manure of BGP1 and activated sludge of WWTP1 shared identical amino acid sequences and represented both OXA-104, which was reported for *A. baumannii* strain 571B5-12EESBL isolated previously from a German biogas plant input sample (Pulami et al., 2020). Isolates from raw and digested manure of BGP6 shared OXA-91 and OXA-385 with *A. baumannii* strains KPC-SM-17a, isolated earlier from a German biogas plant, and a chicken isolate (Pulami et al., 2020; Wilharm et al., 2017). Similarly, isolates from the untreated wastewater of WWTP1 (influent) shared OXA type OXA-431 (identical only at the protein level) reported in avian isolates. Isolates from untreated wastewater, activated sludge, and the effluent of the WWTP1 and the untreated wastewater of WWTP2 shared OXA-106, which was previously found in *A. baumannii* strain 571B5-12EESBL isolated from a German biogas plant input sample (Pulami et al., 2020; Fig. 4).

Based on the Pasteur MLST scheme 23 novel STs [three from BGP2 and BPG6 (ST1599, ST1600, and ST1601), 15 from WWTP1 (ST1582 to ST1596), and five from WWTP2 and WWTP3 (ST1460, ST1461, ST1462, ST1463, and ST1464)] and 12 previously described STs [ST917, ST1027 from BGP1; ST182, ST221, ST367, and ST1049 from WWTP1; ST25, ST79, ST203, and ST427 from WWTP2; ST193 from WWTP3; ST2 from both, WWTP2 and 3] were determined (Fig. 5).

The calculated MST based on Pasteur MLST data showed the relatedness of *A. baumannii* isolates from this study with STs from WWTP receiving hospital wastewater (Higgins et al., 2018), birds (Wilharm et al., 2017), animals (Rafei et al., 2015; Klotz et al., 2019), biogas plant samples (Pulami et al., 2020), and clinically relevant STs from the pubMLST database (Fig. 5). Few STs (ST917, ST1027, and ST1049) from BGP1, BGP6, and WWTP1 were identical to those already reported from animals, however, three STs (ST2, ST25, and ST79) from WWTP2 and 3, were identical to those reported previously in clinics and WWTPs receiving hospital wastewater (Fig. 5). Isolates representing ST2, ST25, and ST79 belonged to the clonal complexes CC2, CC25, and CC79 (Fig. 5). However, ST1460, ST1462, ST1582, ST1584, ST1600, ST1587, ST1589, ST1591, ST1593, ST1599, and ST1595 from this study were SLVs of ST57, ST837, ST126, ST163, ST309, ST46, ST132, ST1303, ST139, ST462, and ST25, and belonged to CC57, CC837, CC126, CC163, CC309, CC149, CC132, CC1303, CC139, CC462, and CC25, respectively (Fig. S4; Table S6, Supporting Information). Comparison between MLST and $bla_{\text{OXA-51-like}}$ typing showed that isolates belonging to the same clonal complex carried the same $bla_{\text{OXA-51-like}}$ gene variant (Fig. 5).

3.6. Antibiotic susceptibility and carbapenem-resistance genes of Acinetobacter sp. isolates

Most of the *A. baumannii* isolates (eight out of nine) from livestock were susceptible to all tested antibiotics, only one isolate of digested manure of BGP6 showed a resistance to imipenem (Fig. 6). The majority of the *A. baumannii* isolates of the rural WWTP (WWTP1; 24 out of 26) were also susceptible to all tested antibiotics. Only one isolate from dewatered sludge of WWTP1 showed an intermediate phenotype to ceftazidime and piperacillin, and one isolate of the effluent of WWTP1 was resistant to colistin. None of the isolate contained one of the tested carbapenemase genes.

In contrast, only nine out of 17 *A. baumannii* isolates from the two urban WWTPs (WWTP2 and 3) were susceptible to all tested antibiotics. The remaining eight isolates of the urban WWTPs, six from untreated wastewater,

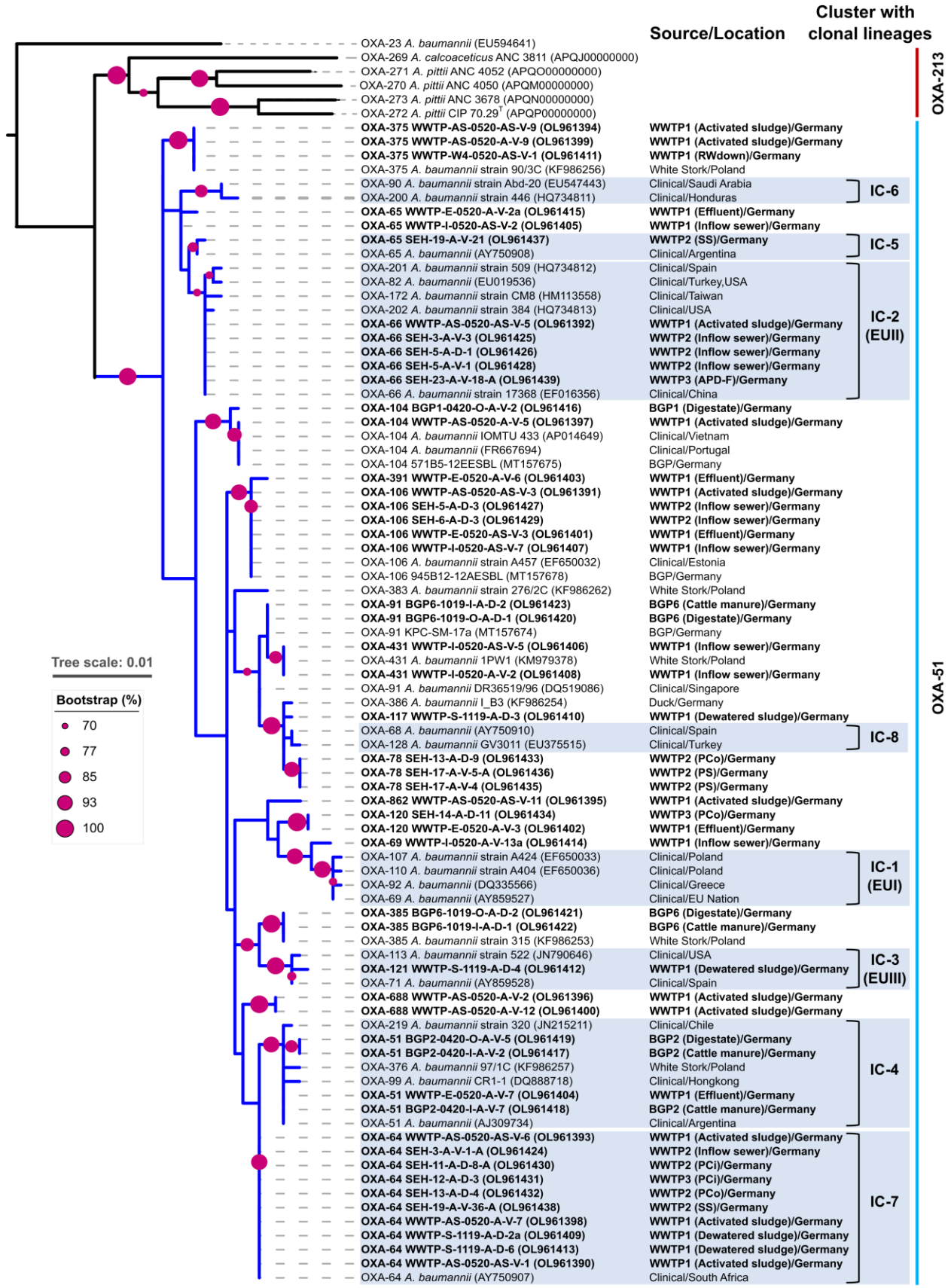


Fig. 4. Phylogenetic correlation of complete nucleotide sequence of $bla_{\text{OXA-51-like}}$ genes from this study with international clonal lineages, clinical, avian and biogas strains of *A. baumannii* calculated by ML method. GenBank accession numbers were given in parentheses. Bootstrap values >70 % (100 resamplings) were indicated by red dots. Bar: 0.01 substitutions per nucleotide position. Strain from this study were highlighted in bold. WWTP: Wastewater treatment plant; BGP, Biogas plant; IC, International clonal lineages; EU, European clonal lineages.

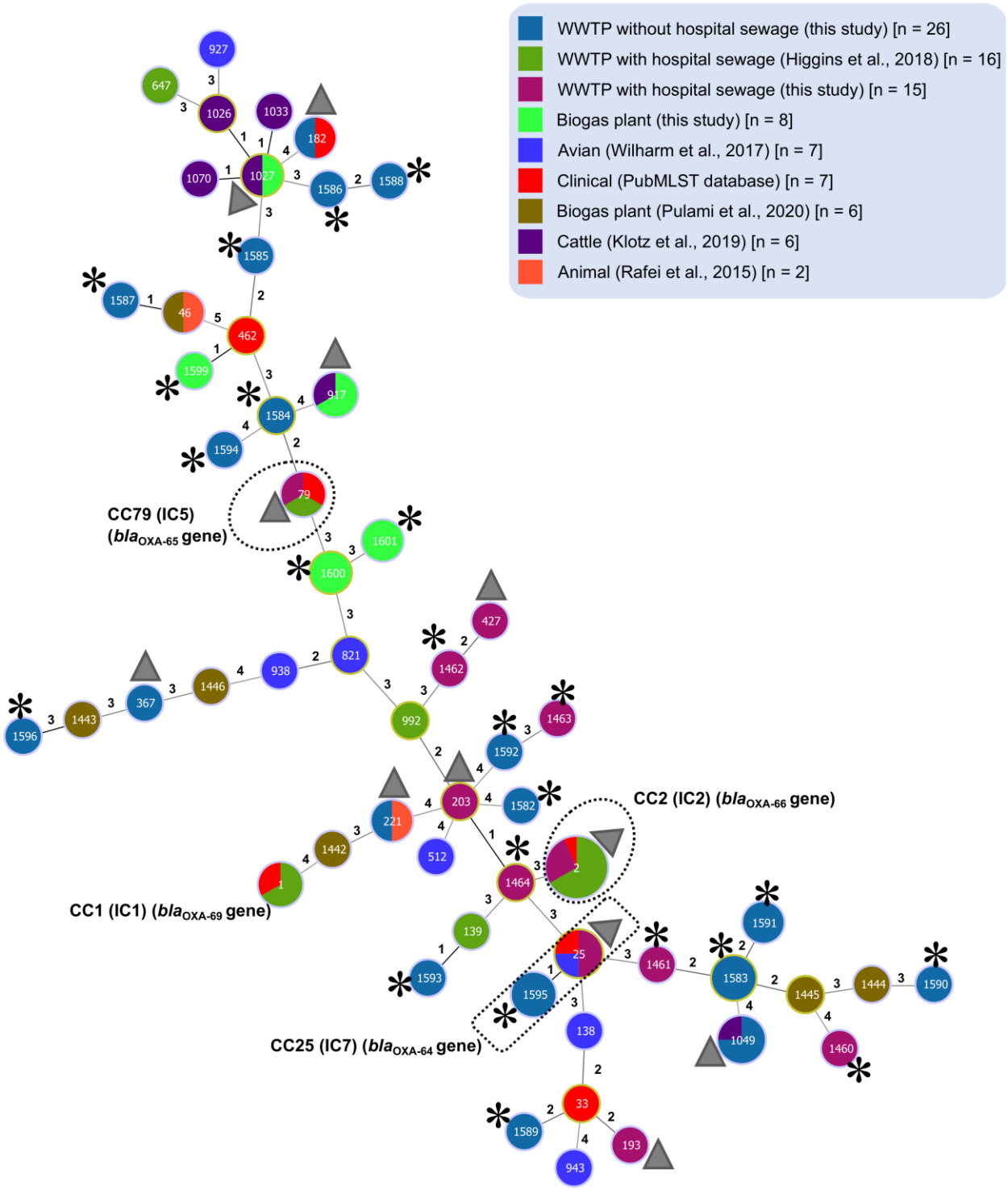
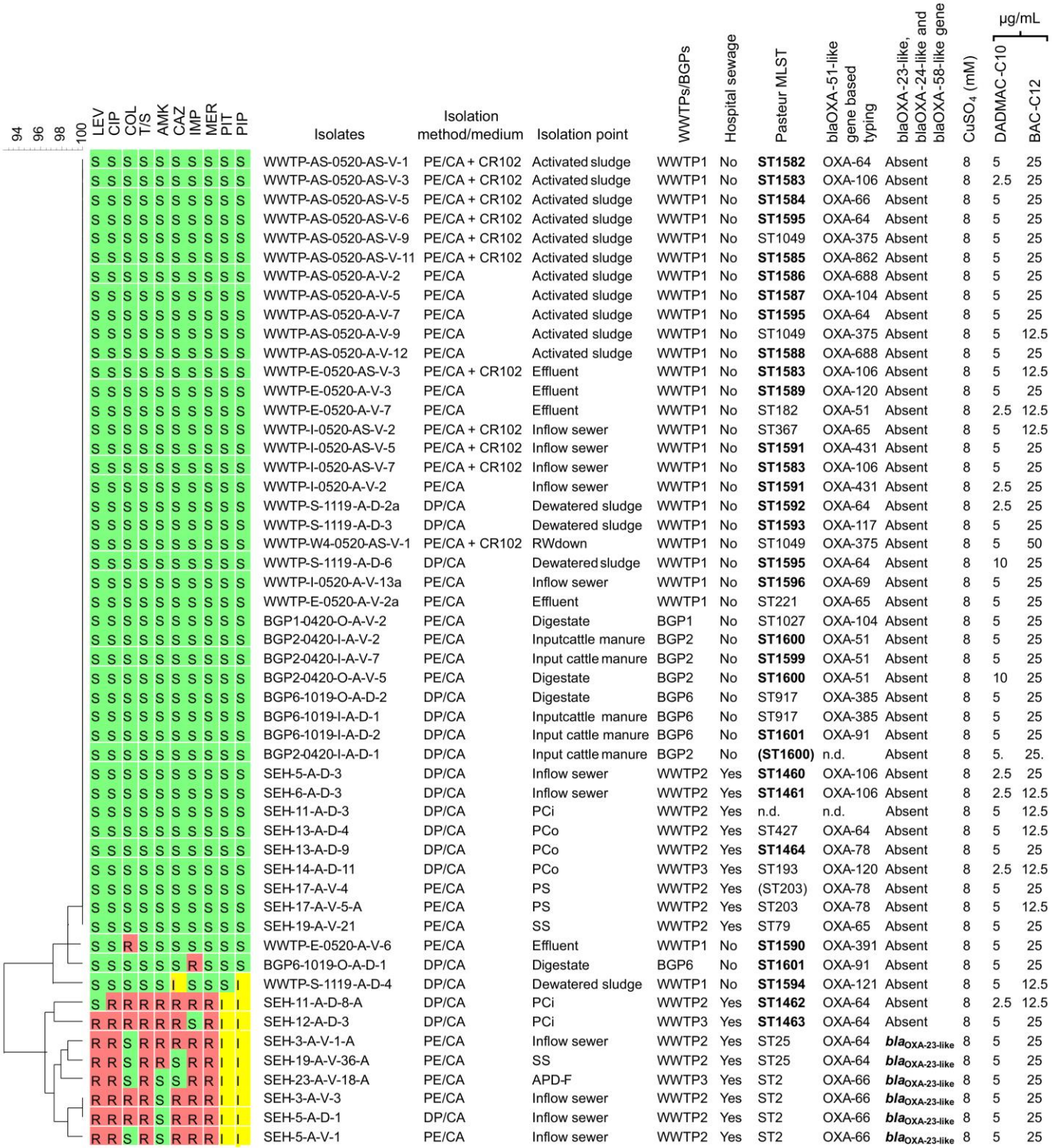


Fig. 5. Pasteur MLST scheme based minimum spanning tree (MST) showing the relatedness of *A. baumannii* isolates from this study with the STs from a wastewater treatment plant receiving hospital sewage (Higgins et al., 2018), avian isolates (Wilharm et al., 2017), animals (Rafei et al., 2015; Klotz et al., 2019), biogas plants (Pulami et al., 2020), and clinically related STs (PubMLST database) calculated by goeBURST algorithm (Phyloviz version 2.0). Numbers along the line indicated the variation among the seven loci between two connected strains. Asterisks and filled triangles indicated the novel and previously described STs identified in this study. CC: Clonal complex, IC: International clonal lineage, n: number of isolates. STs were represented in specific colour codes based on different isolation sources. Circle size was directly proportional to number of the representative ST types. Number of isolates: [n].

the inflow of the primary clarifier and secondary sludge of WWTP2, and two from the inflow of the primary clarifier and anaerobic post-digestor filtrate of WWTP3, showed resistance towards at least five tested antibiotics. All eight *A. baumannii* isolates showed resistance to ciprofloxacin, trimethoprim/sulfamethoxazole, and meropenem (Fig. 6; Table S7, Supporting Information). There was no distinct difference in the occurrence of resistant or sensitive isolates in different studied WWTP compartments. Inhibition of the meropenem resistance in the presence of EDTA confirmed a metallo-beta-lactamase as resistant determinant. Multiplex-PCR screening showed the presence of a $bla_{\text{OXA-23-like}}$ gene in six of the meropenem resistant



LEV	CIP	COL	T/S	AMK	CAZ	IMP	MER	PIT	PIP	Isolates	Isolation method/medium	Isolation point	WWTPs/BGPs	Hospital sewage	Pasteur MLST	blaOXA-51-like gene based typing	blaOXA-23-like, blaOXA-24-like and blaOXA-58-like gene	$CuSO_4$ (mM)	µg/mL DADMAC-C10	µg/mL BAC-C12
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-1	PE/CA + CR102	Activated sludge	WWTP1	No	**ST1582**	OXA-64	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-3	PE/CA + CR102	Activated sludge	WWTP1	No	**ST1583**	OXA-106	Absent	8	2.5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-5	PE/CA + CR102	Activated sludge	WWTP1	No	**ST1584**	OXA-66	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-6	PE/CA + CR102	Activated sludge	WWTP1	No	**ST1595**	OXA-64	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-9	PE/CA + CR102	Activated sludge	WWTP1	No	ST1049	OXA-375	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-AS-V-11	PE/CA + CR102	Activated sludge	WWTP1	No	**ST1585**	OXA-862	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-A-V-2	PE/CA	Activated sludge	WWTP1	No	**ST1586**	OXA-688	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-A-V-5	PE/CA	Activated sludge	WWTP1	No	**ST1587**	OXA-104	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-A-V-7	PE/CA	Activated sludge	WWTP1	No	**ST1595**	OXA-64	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-A-V-9	PE/CA	Activated sludge	WWTP1	No	ST1049	OXA-375	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	WWTP-AS-0520-A-V-12	PE/CA	Activated sludge	WWTP1	No	**ST1588**	OXA-688	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-E-0520-AS-V-3	PE/CA + CR102	Effluent	WWTP1	No	**ST1583**	OXA-106	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	WWTP-E-0520-A-V-3	PE/CA	Effluent	WWTP1	No	**ST1589**	OXA-120	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-E-0520-A-V-7	PE/CA	Effluent	WWTP1	No	ST182	OXA-51	Absent	8	2.5	12.5
S	S	S	S	S	S	S	S	S	S	WWTP-I-0520-AS-V-2	PE/CA + CR102	Inflow sewer	WWTP1	No	ST367	OXA-65	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	WWTP-I-0520-AS-V-5	PE/CA + CR102	Inflow sewer	WWTP1	No	**ST1591**	OXA-431	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-I-0520-AS-V-7	PE/CA + CR102	Inflow sewer	WWTP1	No	**ST1583**	OXA-106	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-I-0520-A-V-2	PE/CA	Inflow sewer	WWTP1	No	**ST1591**	OXA-431	Absent	8	2.5	25
S	S	S	S	S	S	S	S	S	S	WWTP-S-1119-A-D-2a	DP/CA	Dewatered sludge	WWTP1	No	**ST1592**	OXA-64	Absent	8	2.5	25
S	S	S	S	S	S	S	S	S	S	WWTP-S-1119-A-D-3	DP/CA	Dewatered sludge	WWTP1	No	**ST1593**	OXA-117	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-W4-0520-AS-V-1	PE/CA + CR102	RWdown	WWTP1	No	ST1049	OXA-375	Absent	8	5	50
S	S	S	S	S	S	S	S	S	S	WWTP-S-1119-A-D-6	DP/CA	Dewatered sludge	WWTP1	No	**ST1595**	OXA-64	Absent	8	10	25
S	S	S	S	S	S	S	S	S	S	WWTP-I-0520-A-V-13a	PE/CA	Inflow sewer	WWTP1	No	**ST1596**	OXA-69	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	WWTP-E-0520-A-V-2a	PE/CA	Effluent	WWTP1	No	ST221	OXA-65	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP1-0420-O-A-V-2	PE/CA	Digestate	BGP1	No	ST1027	OXA-104	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP2-0420-I-A-V-2	PE/CA	Inputcattle manure	BGP2	No	**ST1600**	OXA-51	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP2-0420-I-A-V-7	PE/CA	Input cattle manure	BGP2	No	**ST1599**	OXA-51	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP2-0420-O-A-V-5	PE/CA	Digestate	BGP2	No	**ST1600**	OXA-51	Absent	8	10	25
S	S	S	S	S	S	S	S	S	S	BGP6-1019-O-A-D-2	DP/CA	Digestate	BGP6	No	ST917	OXA-385	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP6-1019-I-A-D-1	DP/CA	Inputcattle manure	BGP6	No	ST917	OXA-385	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP6-1019-I-A-D-2	DP/CA	Input cattle manure	BGP6	No	**ST1601**	OXA-91	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	BGP2-0420-I-A-D-1	DP/CA	Input cattle manure	BGP2	No	**(ST1600)**	n.d.	Absent	8	5.	25.
S	S	S	S	S	S	S	S	S	S	SEH-5-A-D-3	DP/CA	Inflow sewer	WWTP2	Yes	**ST1460**	OXA-106	Absent	8	2.5	25
S	S	S	S	S	S	S	S	S	S	SEH-6-A-D-3	DP/CA	Inflow sewer	WWTP2	Yes	**ST1461**	OXA-106	Absent	8	2.5	12.5
S	S	S	S	S	S	S	S	S	S	SEH-11-A-D-3	DP/CA	PCi	WWTP2	Yes	n.d.	n.d.	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	SEH-13-A-D-4	DP/CA	PCo	WWTP2	Yes	ST427	OXA-64	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	SEH-13-A-D-9	DP/CA	PCo	WWTP2	Yes	**ST1464**	OXA-78	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	SEH-14-A-D-11	DP/CA	PCo	WWTP3	Yes	ST193	OXA-120	Absent	8	2.5	12.5
S	S	S	S	S	S	S	S	S	S	SEH-17-A-V-4	PE/CA	PS	WWTP2	Yes	(ST203)	OXA-78	Absent	8	5	25
S	S	S	S	S	S	S	S	S	S	SEH-17-A-V-5-A	PE/CA	PS	WWTP2	Yes	ST203	OXA-78	Absent	8	5	12.5
S	S	S	S	S	S	S	S	S	S	SEH-19-A-V-21	PE/CA	SS	WWTP2	Yes	ST79	OXA-65	Absent	8	5	25
S	S	R	S	S	S	S	S	S	S	WWTP-E-0520-A-V-6	PE/CA	Effluent	WWTP1	No	**ST1590**	OXA-391	Absent	8	5	25
S	S	S	S	S	S	R	S	S	S	BGP6-1019-O-A-D-1	DP/CA	Digestate	BGP6	No	**ST1601**	OXA-91	Absent	8	5	25
S	S	S	S	S	I	S	S	S	I	WWTP-S-1119-A-D-4	DP/CA	Dewatered sludge	WWTP1	No	**ST1594**	OXA-121	Absent	8	5	12.5
S	R	R	R	R	R	R	R	I	I	SEH-11-A-D-8-A	DP/CA	PCi	WWTP2	Yes	**ST1462**	OXA-64	Absent	8	2.5	12.5
R	R	R	R	R	R	S	R	I	I	SEH-12-A-D-3	DP/CA	PCi	WWTP3	Yes	**ST1463**	OXA-64	Absent	8	5	25
R	R	S	R	R	R	R	R	I	I	SEH-3-A-V-1-A	PE/CA	Inflow sewer	WWTP2	Yes	ST25	OXA-64	***bla*$_{OXA-23-like}$**	8	5	25
R	R	S	R	R	S	R	R	I	I	SEH-19-A-V-36-A	PE/CA	SS	WWTP2	Yes	ST25	OXA-64	***bla*$_{OXA-23-like}$**	8	5	25
R	R	S	R	S	S	R	R	I	I	SEH-23-A-V-18-A	PE/CA	APD-F	WWTP3	Yes	ST2	OXA-66	***bla*$_{OXA-23-like}$**	8	5	25
R	R	R	R	S	R	R	R	I	I	SEH-3-A-V-3	PE/CA	Inflow sewer	WWTP2	Yes	ST2	OXA-66	***bla*$_{OXA-23-like}$**	8	5	25
R	R	R	R	S	R	R	R	I	I	SEH-5-A-D-1	DP/CA	Inflow sewer	WWTP2	Yes	ST2	OXA-66	***bla*$_{OXA-23-like}$**	8	5	25
R	R	S	R	S	R	R	R	I	I	SEH-5-A-V-1	PE/CA	Inflow sewer	WWTP2	Yes	ST2	OXA-66	***bla*$_{OXA-23-like}$**	8	5	25

Fig. 6. Dendrogram based on UPGMA hierarchical clustering method calculated in BioNumerics v8 (Applied Maths, Belgium). All *A. baumannii* isolates were typically clustered based on their resistance categorical values [absent value = 0, susceptible (S) = 1, intermediate (I) = 2, and resistant (R) = 3] using a categorical coefficient, which treats different values as different states. Antibiotic susceptibility profile of each isolate was converted into resistance categorical value in BioNumerics v8. The colors in the comparison window correspond to the colour of each antibiotic category (S, I, R). Novel STs (Pasteur MLST) were highlighted in bold. MIC, Minimum inhibitory concentration; $CuSO_4$, copper sulfate; BAC-C12, Benzyldimethyldodecylammonium chloride; DADMAC-C10, Didecyldimethylammonium chloride; PE, pre-enrichment; DP, direct plating; CA, ChromAgar™*Acinetobacter* partially supplemented with CR102, ChromAgar MDR supplement; BGPs, Biogas plants; WWTPs, Wastewater treatment plants, APD-F, Anaerobic post digestor filtrate; PCi, Inflow primary clarifier; PCo, Outflow primary clarifier; PS, primary sludge; SS, secondary sludge; RWdown, river water downstream. n.d., Not determined:. Parital 16S rRNA gene-based similarity and MLST types in parentheses are related to isolates identified based on identical fingerprinting patterns (BOX-PCR).

A. baumannii isolates. The isolates were asigned to ST2 ($n = 4$) and ST25 ($n = 2$). All other *A. baumannii* isolates were negative for the presence of additional carbapenemase genes beside the intrinsic $bla_{\text{OXA-51-like}}$ gene.

In addition, 27 isolates from WWTP2 and 3 assigned to the non-*baumannii* ACB complex were subjected to antibiotic susceptibility testing (Fig. S5, Supporting information). Ten out of the 27 isolates were at least resistant to one tested antibiotic. In case of WWTP2, isolates were resistant to one (two isolates, imipenem) or both (one isolate) tested carbapenems. These isolates were obtained from untreated wastewater and secondary sludge. In WWTP3, isolates from the inflow of the primary clarifier were resistant to one (trimethoprim/sulfamethoxazole), three (two tested quinolones and trimethoprim/sulfamethoxazole), or even seven of the different tested antibiotics (Fig. S5). The multi-resistant isolate contained beside the quinolone resistance, a resistance to colistin, amikacin, ceftazidime, and the tested carbapenems. Also, in WWTP3 one isolate from the inflow of the primary clarifier was resistant to ciprofloxacin, and two isolates from the secondary sludge were resistant to both carbapenems (Fig. S5). Meropenem resistant isolates showed an inhibition of the meropenem resistance in the presence of EDTA indicating the expression of a metallo-beta-lactamase as resistance mechanism. Three of the meropenem resistant isolates, SEH-20-A-V-6-A and SEH-20-A-V-7-A (secondary sludge isolates, WWTP-2) and SEH-3-A-V-2-A (sewer isolate, WWTP-3), contained a $bla_{\text{OXA-24-like}}$ gene.

3.7. Heavy metal ion (Cu^{2+}) and QAAC tolerance of *A. baumannii* isolates

All 52 *A. baumannii* isolates grew on MH agar containing 4 mM $CuSO_4$, but not in the presence of 8 mM $CuSO_4$ (Fig. 6; Fig. S6, Supporting Information). The MIC values for BAC-C12 were in the range of 12.5 and 25 μg mL^{-1} and for DADMAC-C10 in the range of 2.5 to 10 μg mL^{-1}. No differences in MIC values were obtained for *A. baumannii* isolates cultured from the different livestock and WWTPs samples (Fig. 6).

4. Discussion

4.1. Detection of *Acinetobacter* in livestock sources

There are only few studies available that investigated the diversity and resistance patterns of *Acinetobacter* from non-human sources including the livestock environment (Wareth et al., 2021; Klotz et al., 2019; Pulami et al., 2020). Recently, we already cultured ACB complex isolates including six *A. baumannii* isolates from raw manure and digested manure of other German biogas plants with mesophilic and thermophilic operating conditions (Pulami et al., 2020). The isolates represented non-clinical STs and did not carry antimicrobial resistances to clinically relevant antibiotics. In contrast to our current study, we had not used a specific medium for the systematic analysis of the abundance of *Acinetobacter*.

Klotz et al. (2019) studied the phylogenetic diversity and resistance patterns of bovine *A. baumannii* strains collected in the same area where our studied farms were located. In accordance to our data, cattle were associated with a diverse population of *A. baumannii* strains, but did not represent a reservoir of nosocomial strains. The highest frequency of *A. baumannii* was determined in dairy cows (21.1 %) (Klotz et al., 2019). In the present study we determined *A. baumannii* in raw and digested manure obtained from dairy farms.

4.2. Detection of (potential meropenem resistant) *Acinetobacter* in WWTPs

Specific abundance trends of culturable *Acinetobacter* were detected by the comparative analysis of the different WWTPs. The detection frequency of viable total *Acinetobacter* and viable potential meropenem resistant *Acinetobacter* was lower in the rural compared to the urban WWTPs. The load of viable *Acinetobacter* including *A. baumannii* was higher in the untreated wastewater (influent) of the urban WWTPs, which contained wastewater from veterinarian and human clinics, than the untreated wastewater of the rural WWTP. While total and potential meropenem resistant *Acinetobacter* could be cultured from one of the effluent samples of the rural WWTP, no *Acinetobacter* were cultured from both effluent samples of the urban systems. Repeated sampling was not yet performed but may lead to different outcomes. In contrast to our study, other studies reported the cultivation of meropenem resistant *Acinetobacter* in untreated and in reduced abundance also from treated wastewater (effluents) indicating a reduction but no complete elimination of *Acinetobacter* in treated wastewater (Hrenovic et al., 2016; Goic-Barisic et al., 2016, 2017; Müller et al., 2018; Higgins et al., 2018; Hubeny et al., 2022). Because *Acinetobacter* were not cultured from the effluent samples of our study, the *Acinetobacter* species determined downstream of the effluent discharge may come from another source.

Several studies which investigated the spread of ARBs from WWTPs were focused on the effluent and the receiving rivers, but did not consider the presence of ARBs in the digested sewage sludge (Hrenovic et al., 2016; Goic-Barisic et al., 2016, 2017; Müller et al., 2018). However digested sewage sludge represents the biosolids that are used as fertilizer on agricultural fields. Here we could show that the effluent (treated wastewater) is not the key hotspot for *Acinetobacter* transmission into the environment. *Acinetobacter* spp. were detected in the inflow and outflow of the primary clarify, primary sludge, in activated sludge, and secondary sludge. Even after anaerobic sewage sludge digestion viable *Acinetobacter* were still detected. The presence of *Acinetobacter* spp. in anaerobically digested sludge was already reported by Higgins et al. (2018) and Guardabassi et al. (2002). Higgins et al. (2018) suggested that the application of an alkaline lime treatment however could avoid the spread of *Acinetobacter* via sludge based fertilizers into the environment.

Based on the presence of *Acinetobacter* in activated sludge and digested sewage sludge, Higgins et al. (2018) suggested the incorporation of *Acinetobacter* into activated sludge flocs. This was already confirmed by in situ hybridization studies with an *Acinetobacter* specific 16S rRNA probe by Wagner et al. (1994). These authors showed that 3 and 7 % of the stained bacterial cells attached to activated sludge flocs of aerobic and anaerobic treatment tanks represented *Acinetobacter* cells. Phuong et al. (2012) determined by in situ hybridization studies performed with the same *Acinetobacter* specific probe the highest population of *Acinetobacter* in settled sludge (6.7 % of the stained cells) and settled sewage of the primary setting tank (4 %). The authors showed the intergeneric co-aggregation of the non-flocculating *Acinetobacter* isolates with several floc forming bacteria (e.g., *Pseudomonas*) isolated from activated sludge. This intergeneric attachment and floc-formation may have a strong impact on horizontal gene transfer leading to the exchange of ARGs within and among different genera. As shown recently anaerobically digested sewage sludge (biosolids) collected from German WWTPs contain a broad range of pollutants including antibiotics, disinfectants, and heavy metals (Wolters et al., 2022), which can trigger horizontal gene transfer. However, detailed experimental proofs are not yet given.

4.3. High diversity of cultured *A. baumannii* in treated and untreated livestock and human wastewater

A high diversity of *Acinetobacter* asigned to non-clinically relevant and novel *A. baumannii* STs were isolated from livestock and the rural WWTP (not receiving clinical wastewater). This indicated that non-clinical environments harbour a different diversity of *A. baumannii* which were phylogenetically distinct to those circulating in hospital/veterinary settings (Zander et al., 2012; Wareth et al., 2019, 2020a). Most of those *A. baumannii* isolates were susceptible to the tested clinically relevant antibiotics. This was also confirmed by the previous findings of phylogenetically diverse and susceptible *Acinetobacter* spp. in various non-clinical impacted environments such as biogas plants (Pulami et al., 2020), avian (Wilharm et al., 2017), bovine (Klotz et al., 2019), milk powder (Cho et al., 2018; Wareth et al., 2021), meat and manure from cattle (Rafei et al., 2015), and rural WWTPs (Müller et al., 2018). Some of the STs (ST917, ST1027, and ST1049) cultured from livestock and the rural WWTP were previously reported from cattle of the same region in Germany (Klotz et al., 2019).

In contrast, several clinically relevant *A. baumannii* ST2, ST25, and ST79, which belong to globally distributed IC lineages IC2, IC7, and IC5 were cultured only from the urban WWTPs received veterinarian and human clinical wastewater. Sequence types of these international lineages were associated with outbreaks in hospital settings in Europe and across the globe (Di Popolo et al., 2011; Karah et al., 2011; Chagas et al., 2014; Sahl et al., 2015; Molter et al., 2016; Da Silva et al., 2018; Hamidian and Nigro, 2019; Camargo et al., 2020). However, those STs are not limited to humans and clinical settings; for instance, ST2 was also detected in pets admitted to veterinary clinics in Germany (Ewers et al., 2017), isolated from sheep (infected with virulent ST2 strain causing pneumonia) in Pakistan (Linz et al., 2018), and a croatian WWTP receiving hospital wastewater (Higgins et al., 2018). Similarly, ST25 was among the dominant STs found in animals treated in veterinary hospital in Switzerland (Püntener-Simmen et al., 2019). ST79 was reported in a WWTP which received hospital wastewater (Higgins et al., 2018) and a river located in the urban area in Brazil (Turano et al., 2016). In our study it was indicated that hospital/veterinary settings seemed to be the primary source of clinical clonal groups (STs) and their release into the environment.

All *A. baumannii* isolates, but no other *Acinetobacter* isolates of this study harbored complete and uninterrupted $bla_{\text{OXA-51-like}}$ genes, which are intrinsic to *A. baumannii* and had been rarely reported in isolates not belonging to *A. baumannii* (Lee et al., 2012). The $bla_{\text{OXA-51-like}}$ genes of *A. baumannii* isolates encoded amino acid sequence variants such as OXA-69, OXA-66, OXA-51, OXA-65, and OXA-64, which have been reported for clinical *A. baumannii* lineages IC-1, −2, −4, −5, and −7 (Zander et al., 2012). However, nucleotide sequences of the OXA-51 and OXA-65 variants obtained from livestock, the rural and urban WWTPs, were not identical to those of strains of clinical lineages IC-4 and IC-5 (Fig. 4). Similar nucleotide sequence variants for OXA-51, OXA-65, and OXA-69 were also reported for *A. baumannii* strains isolated from avian (wild stork) (Wilharm et al., 2017). In clinical *A. baumannii* isolates the integration of IS*Aba1* element into the promoter region of $bla_{\text{OXA-51-like}}$ genes promotes the β-lactam hydrolyzing activity of this weak β-lactamase (overexpression of resistance genes; Turton et al., 2006; Zarrilli et al., 2013). Our strains from livestock and WWTP samples lacked the IS*Aba1* insertion upstream of $bla_{\text{OXA-51-like}}$ genes. The absence of the IS*Aba1* insertion in wastewater derived isolates was also reported by Higgins et al. (2018). In contrast, Hubeny et al. (2022) reported the presence of IS*Aba1*/$bla_{\text{OXA-51}}$ complexes in *A. baumannii* isolates from treated wastewater.

The finding that *A. baumannii* isolates belonging to the same CCs expressing identical OXA-51-like protein variants is in accordance with previous findings for isolates from human (Evans et al., 2008; Pournaras et al., 2014), and non-clinical environments (Rafei et al., 2015; Higgins et al., 2018). We determined that identical OXA-51-like protein variants were shared by multiple distinct STs, like ST1582, ST1592, and ST1595 from activated and dewatered sludge of WWTP1 that shared OXA-64, ST1599 and ST1600 from raw and digested manure of BGP2 and ST182 from effluent of WWTP1 that shared OXA-51, ST1588 and ST1586 from activated sludge of WWTP1 that shared OXA-68, ST1027 from digested manure of BGP1, and ST1587 from activated sludge of WWTP1 that shared OXA-104, and ST367 and ST221 from influent and effluent of WWTP1 that shared OXA-65. In line with our observations, it was previously reported that some distinct *A. baumannii* STs shared the same variant of the OXA-51-like protein, i.e. *A. baumannii* strains from chicken, goose, turkey, and wild white storks originating from Germany and Poland (Wilharm et al., 2017), some strains of domesticated animals and their products, and artesian well water in Lebanon (Rafei et al., 2015). Overall these findings indicated the convergent evolution of the $bla_{\text{OXA-51-like}}$ gene which has to be further evaluated.

4.4. Antimicrobial resistance of Acinetobacter in treated and untreated livestock and human wastewater

In contrast to the livestock and the rural WWTP, 47 % (8/17) of the *A. baumannii* and other ACB isolates from the two urban WWTPs showed phenotypic resistance towards multiple antibiotics beside carbapenem resistance. Six of these multidrug resistant (MDR) *A. baumannii* isolates were identified as clinical ST2 and ST25 while two isolates represented novel STs (ST1462 and ST1463). According to the recommendation by the Commission for Hospital Hygiene and Infection Prevention (KRINKO) at the Robert Koch Institute, Germany, the *Acinetobacter* isolates can be classified as 3 or 4MRGNs (multidrug resistant gram negatives) (Wendt et al., 2012; Kaase, 2016). According to this classification system 3MRGN *Acinetobacter* strains are resistant to piperacillin, third generation cephalosporins, and fluoroquinolones and 4MRGN *Acinetobacter* strains are additionally resistance to carbapenems. Among the MDR *A. baumannii* isolates of ST2, three isolates of untreated wastewater of one of the urban WWTPs were considered as 4MRGN and one isolate cultured from the post-anaerobic digestor filtrate of WWTP3 showed resistance to ciprofloxacin and meropenem and was intermediate resistant to piperacillin. Similarly, isolates of ST25 of untreated wastewater and secondary sludge of WWTP2 and the novel STs, ST1462 from inflow to primary clarifier of WWTP2 and ST1463 of the inflow to the primary clarifier of WWTP3, were defined as 3MRGN and/or 4MRGN. The carbapenem-resistant ST2 and ST25 isolates carried a $bla_{\text{OXA-23-like}}$ carbapenemase gene. The $bla_{\text{OXA-23-like}}$ gene was determined as main resistance determinant among carbapenem-resistant *A. baumannii* isolates from clinical environments in Germany and other countries (Zowawi et al., 2015; Rieber et al., 2017; Palavecino et al., 2022). In a German study of *A. baumannii* isolates from milk powder and human sources $bla_{\text{OXA-23-like}}$ genes were only detected in isolates of human, but not of milk powder sources (Cho et al., 2018). Among the human source isolates the authors also determined *A. baumannii* isolates of ST2 (Pasteur Scheme). Strains of this ST were also responsible for previous nosocomial outbreaks in German hospitals. *A. baumannii* isolates of ST2 carrying a $bla_{\text{OXA-23-like}}$ gene were also detected previously by Higgins et al. (2018) in the urban WWTP of Zagreb (Croatia). Non-*baumannii* ACB complex isolates, which were cultured from the urban WWTP system, were also classified as 4MRGNs (Fig. S5). This included an isolate next related to the clinically relevant *A. pittii* (Wong et al., 2017) which was cultured from the inflow of the primary clarifier of WWTP3. This and two other carbapenem-resistant non-baumannii ACB complex isolates carried $bla_{\text{OXA-24-like}}$ carbapenemase genes. Of the 4MRGN *A. baumannii* isolates mentioned above, five of them showed additional resistance to colistin (COL; Figs. 6; S5). Infection cases with 3MRGN and 4MRGN *Acinetobacter* strains are increasingly reported in Germany (Siemers et al., 2014; Hauri et al., 2015; Huenges et al., 2016; Wareth et al., 2020a). Beside *A. baumannii* other species of the ACB complex, especially *A. pittii,* were isolated in high frequencies from patients of clinical settings in Germany (Schleicher et al., 2013). MDR *A. baumannii* of the IC1 and IC2 (formerly European clones I and II) were also considered as endemic pathogens within veterinary clinics in Germany (Zordan et al., 2011). Apart from clinics, *A. baumannii* strains of the IC1 and IC2 lineages resistant to quinolone, carbapenem, and colistin were cultured from influent, effluent, activated sludge, and digested sludge from an urban WWTP in Croatia which received hospital wastewater and had a mesophilic anaerobic digestion process (Higgins et al., 2018). Even though *A. baumannii* isolates were not cultured from effluent samples of WWTP2 and WWTP3, the viable 3MRGN isolates related to IC2 (ST2) and IC7 (ST25) were isolated from the anaerobic post-digestor filtrate and secondary sludge after anaerobic treatment in WWTPs of our study. Since, treated sewage sludge is used as biosolids for the fertilization of agricultural fields (Roskosch and Heidecke, 2019), the risks of spread of clinically relevant MRGN representing *A. baumannii* strains of ST2 and ST25 and *A. pittii* related isolates all carrying clinically relevant carbapenemase genes ($bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-24-like}}$) into the agricultural environment cannot be excluded. Especially the resistance to carbapenems and colistin is of concern because these drugs are among few antibiotics used against infections with MDR bacteria including *Acinetobacter* (Falagas and Kasiakou, 2005; Wong et al., 2017). The resistance of *Acinetobacter* to these antibiotics are increasingly reported (Higgins et al., 2010; Göttig et al., 2014; Nowak et al., 2017; Gerson et al., 2019) and also determine in low frequency in the WWTPs of our study.

Interestingly, while differences were obtained for the susceptibility to clinically relevant antibiotics, the susceptibility to disinfectants and copper (heavy metal) were not different among the *A. baumannii* isolates obtained from the different sources. Both kind of compounds (disinfectants and heavy metals) may play an important role for co-selection processes of antibiotic resistances if *Acinetobacter* strains which are released from the different sources into the environment (Rizzo et al., 2013; Imran et al., 2019). More detailed studies are required to understand these processes in detail.

4.5. Survival of Acinetobacter under anoxic conditions

Although *Acinetobacter* are regarded as strictly aerobic bacteria (Towner, 2006), viable *A. baumannii* and *A. pittii* isolates were detected in anaerobically treated manure and digested sewage sludge of the urban WWTPs. It is known that *Acinetobacter* strains are able to accumulate polyphosphates which they may use to gain energy to survive the anaerobic treatment. Polyphosphate gained energy can be vital under anaerobic environmental conditions when these strict aerobic bacteria have limited options to generate energy (Van Groenestijn et al., 1987; Bark et al., 1992). We had previously shown that genes encoding the AMP phosphotransferase and adenylate kinase are present in *A. baumannii* strains obtained from raw and digested manure (Pulami et al., 2020, 2021). These enzymes are involved in the processing of polyphosphates to gain energy (Kämpfer et al., 1992). In addition, wastewater derived *Acinetobacter* formed dormant cells under anoxic conditions in the experimental setup (Dekic et al., 2019). All together our study supported the previous statements that a proper treatment of sewage sludge and digestates generated in BGPs and WWTPs is required to prevent the spread of the obligate aerobic *Acinetobacter* into the environment (Hrenovic et al., 2016; Higgins et al., 2018; Pulami et al., 2020).

4.6. Cultivation bias in risk assessment

The quantitative and also qualitative detection of viable *Acinetobacter* is limited. As pointed out earlier (Higgins et al., 2018), there are no selective cultivation conditions for *Acinetobacter* and more specifically for *A. baumannii*. The use of the selective CHROMagar medium for quantificative cultivation of *Acinetobacter* is time consuming and requires subsequent colony identification because non-target bacteria also grew with an *Acinetobacter* specific phenotype. The reduction of non-target bacteria growth by the addition of cefsulodin (5 mg L^{-1}) partially reduced but partial did not effect the growth of non-target bacteria. Other studies increased the cefsulodin concentration to 15 mg L^{-1} (Goic-Barisic et al., 2016, 2017; Hrenovic et al., 2016; Higgins et al., 2018) but still reported growth of non-target bacteria. In addition the incubation temperature was increased to 42 °C in those studies to further reduce the growth of non-target bacteria. Increased incubation temperature is often recommendation for the selective growth of ESKAPE bacteria from environmental samples (Schreiber et al., 2021). But, it was for example recently shown that the incubation at 42 to 44 °C in the presence of carbapenems can hinder the regrowth of starved *Acinetobacter* from environmental samples (Dekić Rozman et al., 2021). Proteome analysis revealed that impaired membrane integrity and reduced functionality of efflux pumps at the elevated temperature were responsible for the growth deficiency. Dekić Rozman et al. (2021) showed that a preincubation at 36 °C could lead to a recovery and cultivability of *Acinetobacter* at 44 °C in the presence of carbapenems. For that reason, we used a four hours pre-incubation at 37 °C followed by a subsequent incubation at 44 °C if we applied the cultivation at 44 °C (in few cases). Most samples were incubated at 37 °C in this study. The diversity of viable *Acinetobacter* may be larger than obtained because not all *Acinetobacter* species can grow at 37 °C (Krizova et al., 2014, 2015). Although species of the genus *Acinetobacter* that do not show growth at 37 °C lack clinical relevance, they can contribute to the spread of antimicrobial resistances within the environment and back to pathogenic strains. Other studies that used only 30 °C as incubation temperature to culture *Acinetobacter* may have targeted a broader range of the present *Acinetobacter* populations (Guardabassi et al., 2002; Zhang et al., 2009; Hubeny et al., 2022).

Another problem which occurred during the cultivation of meropenem resistant *Acinetobacter* was that most of the *Acinetobacter* isolates which were obtained after enrichment in the presence of meropenem were in pure culture sensitive to carbapenems. Similarly, the lack of phenotypic resistance to carbapenems had been reported for bovine *A. baumannii* isolates, which were able to grow on MH agar supplied with 2 and 4 mg L^{-1} meropenem when swap samples were streaked on agar plates for initial *Acinetobacter* spp. cultivation (Klotz et al., 2019). The repeated streaking and transfer of biomass of these isolates on MH agar plates (without antibiotic) during purification, might have resulted in loss of acquired ARGs and mobile genetic elements (MGEs) to compensate the fitness cost associated with ARGs and MGEs inside the host bacterium (Andersson and Hughes, 2010; Vogwill and MacLean, 2015; Durão et al., 2018). This phenomenon however was not proven yet for the cultivation of *Acinetobacter* in this study or other studies which reported this problem. All together it has to be concluded that the cultivation of *Acinetobacter* from environmental samples is extremely important to understand AMR transmission but still requires some optimization. The outcome of current studies has to be compared with care due to the different incubation conditions which were applied to culture *Acinetobacter*.

5. Conclusion

We showed in a direct comparative study that clinically relevant and antimicrobial resistant *Acinetobacter* are released from urban WWTPs receiving clinical wastewater into the environment, while *Acinetobacter* from livestock (raw and digested manure) and rural WWTPs (no clincal wastewater inflow), lacked clinical relevance (based on MLST) and were susceptible to most antibiotics. More comparative studies are required to understand the behavior of *Acinetobacter* within the studied systems and the receiving environments. Strain specific properties as contribution to floc-formation, particle-attchement, and the selective colonization of specific ecological microniches in receiving aquatic and soil environments must be studied. Considering the high load of pollutants released in parallel to bacteria into those environments, co-selection processes in the different ecological microniches should be investigated in detail. Taking the "One Health Approach" (Adisasmito et al., 2022) as contemporary issue, genome-based comparisons of *Acinetobacter* strains from the studied sources and the receiving environments with clinical strains are required to understand the ongoing evolution of MDR formation in the genus *Acinetobacter*. A current limitation for comparative studies is the low number of available genomes of environmental *Acinetobacter* strains. This was recently pointed out by Roguet et al. (2022) who searched for respective genomes to assemble their sewer derived *Acinetobacter* metagenome data. This confirms the importance of the cultivation of environmental *Acinetobacter* to understand the dissemination of microbial resistances in the environment. All isolates cultured in this study are well characterized and stored in our strain collection to enable future research on these important open research questions.

CRediT authorship contribution statement

PK and SG designed the study layout and received the financial support; DP and SG performed and organized the sampling campaigns; DP did the lab work; SG was responsible for data curation; DP and SG wrote the original draft of the manuscript; PK reviewed and edited the manuscript.

Data availability

All sequence data generated within this study have been submitted to GenBank. All respective sequence accession numbers are given in the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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CHAPTER III

Acinetobacter baumannii in manure and anaerobic digestates of German biogas plants

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Contributions:

SG designed the study. SG and TS organized the samples from German biogas plants in 2012 and 2013. TS performed the cultivation and isolation experiments. DP performed the molecular and physiological tests. TE provided MALDI-TOF data. GW provided the genome sequence data. JB, AG and DP perfromed genome annotation and/or genome sequence-based analyses. PK provided the laboratory facilities. DP and SG wrote the manuscript which was proofed by all co-authors. This project was funded by two Federal Ministry of Education and Research (BMBF)-funded projects, RiskAGuA (02WRS1274A) and ARMIS (5th JPIAMR Joint Call). DP was funded by the Justus Liebig University Giessen (Ph.D. Grand

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RESEARCH ARTICLE

Acinetobacter baumannii in manure and anaerobic digestates of German biogas plants

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One sentence summary: Phylogenetic, phenotypic and genomic characterization of *Acinetobacter* spp. including *A. baumannii* cultured from manure and biogas plant digestates of anaerobically digested manure.

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ABSTRACT

Studies considering environmental multidrug-resistant *Acinetobacter* spp. are scarce. The application of manure on agricultural fields is one source of multidrug-resistant bacteria from livestock into the environment. Here, *Acinetobacter* spp. were quantified by quantitative polymerase chain reaction in manure applied to biogas plants and in the output of the anaerobic digestion, and *Acinetobacter* spp. isolated from those samples were comprehensively characterized. The concentration of *Acinetobacter* 16S ribosomal ribonucleic acid (rRNA) gene copies per g fresh weight was in range of 10^6–10^8 in manure and decreased (partially significantly) to a still high concentration (10^5–10^6) in digestates. 16S rRNA, *gyrB-rpoB* and $bla_{\text{OXA51-like}}$ gene sequencing identified 17 different *Acinetobacter* spp., including six *A. baumannii* strains. Multilocus sequence typing showed no close relation of the six strains with globally relevant clonal complexes; however, they represented five novel sequence types. Comparative genomics and physiological tests gave an explanation how *Acinetobacter* could survive the anaerobic biogas process and indicated copper resistance and the presence of intrinsic beta-lactamases, efflux-pump and virulence genes. However, the *A. baumannii* strains lacked acquired resistance against carbapenems, colistin and quinolones. This study provided a detailed characterization of *Acinetobacter* spp. including *A. baumannii* released via manure through mesophilic or thermophilic biogas plants into the environment.

Keywords: biogas plant; Acinetobacter; anaerobic digestion; whole genome sequencing; manure; antibiotic resistance

INTRODUCTION

The genus *Acinetobacter* represents a heterogeneous group of glucose non-fermentative, catalase-positive, oxidase-negative, aerobic, Gram-negative coccobacilli (Lee *et al.* 2017). Most of the *Acinetobacter* species are considered to be ubiquitous in the environment (Towner 2009). In terms of clinical significance,

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multidrug-resistant *Acinetobacter* belonging to the *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex (ACB complex; Towner 2009) is seen as a major current One Health risk. For the clinically relevant species of *Acinetobacter*, only few data on their environmental niches are available (Berlau *et al.* 1999; Houang *et al.* 2001; Huys *et al.* 2007; Hrenovic *et al.* 2014; Rafei *et al.* 2015; Wilharm *et al.* 2017; Klotz *et al.* 2019).

Currently, *Acinetobacter* spp. are receiving increasing attention as significant opportunistic pathogens and are associated with infections in critically ill patients (Visca, Seifert and Towner 2011). Of these, multidrug-resistant strains of *A. baumannii* are among the most troublesome pathogens globally, and represent one of the ESKAPE pathogens (ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) (Rice 2008). Recently, the World Health Organization (WHO) has for the first time released a list of drug-resistant bacteria that pose the lethal threat to modern medicine and human health—and for which novel antimicrobials are desperately needed. This multidrug-resistant pathogens list is topped by multidrug-resistant phenotype of *A. baumannii* (Willyard 2017). In three decades, this bacterium has evolved from being completely antimicrobial susceptible to mostly multidrug resistant, in parts by expanding a genomic resistance island encoding resistance to several classes of antibiotics, which were horizontally transferred mostly via plasmids, transposons or integrons from bacteria of the genera *Pseudomonas*, *Escherichia* and *Salmonella* (Fournier *et al.* 2006). This expeditious emergence and dissemination of multidrug-resistant *A. baumannii* showed the successful adaptation of this bacterium to selective environmental pressure (Dijkshoorn, Nemec and Seifert 2007; Kempf and Rolain 2012). The resistant strains of *A. baumannii* produce carbapenemase enzymes that are able to hydrolyze carbapenems (β-lactam antibiotics), which are usually considered as 'last-line antibiotics' against resistant pathogens (Perez *et al.* 2010; Papp-Wallace *et al.* 2011; Kempf and Rolain 2012). Carbapenem resistance mostly occurs via class D OXA type serine oxacillinases (bla_{OXA} type, e.g. $bla_{\text{OXA-23}}$) and class B metallo-β-lactamases (MBLs, e.g. New Delhi metallo-β-lactamases, NDM-1), which are largely responsible for clinical outbreaks worldwide (Kempf and Rolain 2012; Zarrilli *et al.* 2013). So far the colistin resistance in *A. baumannii* is mediated by mutations in the two-component system proteins PmrA/B and lipid A synthetic genes (Adams *et al.* 2009; Moffatt *et al.* 2010) and lipid A modification by inclusion of phosphoethanolamine (Qureshi *et al.* 2015). The advent of carbapenem- and colistin-resistant *A. baumannii* is of significant attention for global health, as it heralds an age of limited effective antibiotic choices, and multidrug-resistant *A. baumannii* strains are notorious because of their rapid evolution, increasing prevalence and the ability to cause outbreaks (Perez *et al.* 2010; Kempf and Rolain 2012).

The wide use of antibiotics in humans is one of the primary factors for the rise of resistances (Goossens *et al.* 2005; Costelloe *et al.* 2010; Davies and Davies 2010). Apart from clinical use, antibiotics are largely used in animal husbandry at therapeutic level for disease treatment, accounting for a significant proportion of the antibiotics produced globally (Aarestrup 2005; Chee-Sanford *et al.* 2009; Van Boeckel *et al.* 2015). It has been estimated that ~75% of antibiotics administered to animals are excreted (Chee-Sanford *et al.* 2009). Animal husbandry and manure have been frequently referred to as reservoirs for potentially pathogenic and antibiotic-resistant bacteria and antibiotic resistance genes (Hölzel *et al.* 2010; Friese *et al.* 2013; Marti *et al.* 2013; Zhu *et al.* 2013; Von Salviati *et al.* 2015). Thus, the use of manure as fertilizer in soil can result into dissemination of antibiotic-resistant bacteria/antibiotic resistance genes into the environment (Ghosh and LaPara 2007; Heuer, Schmitt and Smalla 2011; Rahube and Yost 2012; Jechalke *et al.* 2014). Apart from direct field application, manure is also used as carbon source in biogas plants that have been gradually increasing in number throughout Germany. To avoid direct dispense of manure into soil and aquatic ecosystems, anaerobic digestion with partially high process temperatures is considered as sustainable approach resulting in production of biogas and biofertilizers, thereby decreasing the microbial load of the surrounding environments (Bagge, Sahlström and Albihn 2005; Saunders *et al.* 2012; Manyi-Loh *et al.* 2013; Resende *et al.* 2014). Resende *et al.* (2014) identified multidrug-resistant bacterial isolates from the influent and effluent sources of an anaerobic digestion treatment plant for cattle manure, and pleaded for the implementation of sanitary and microbiological safe treatments of manures to avoid unintended consequences to humans, animals and the environment, respectively.

Currently, there are only few studies considering the release of antibiotic resistance genes and culturable antibiotic-resistant bacteria from biogas plants. Schauss *et al.* (2015, 2016) recently showed that extended spectrum β-lactamase (ESBL)-producing *E. coli* were transmitted through mesophilic anaerobic biogas plants, and identified other potentially pathogenic bacterial isolates (including few isolates identified as *Acinetobacter* spp.) from the studied input and output samples. Glaeser *et al.* (2016) reported vancomycin-resistant enterococci (VRE) from the same input and output materials of the biogas plants, indicating that enterococci with vancomycin resistance genes could be released into the environment by application of biogas plant's treated manure as biofertilizers. Wolters *et al.* (2014) reported the transmission of mobile antibiotic resistance plasmids through biogas plants that may be taken up by non-resistant bacteria under changing environmental conditions.

Although the presence of *Acinetobacter* spp. resistant to multiple antibiotics had been investigated in animal husbandry (Wang *et al.* 2012; He *et al.* 2019) and manure (Hrenovic *et al.* 2019), the risk associated with their release into the environment after manure processing in biogas plants has not been investigated so far. The present study thus aimed to determine the abundance of *Acinetobacter* spp. in input (mixed manure) and output (anaerobic digestates) samples of various German biogas plants, and to determine whether or not the anaerobic processing of manure in biogas plants effects the transmission of the aerobic *Acinetobacter* spp. into the environment. Quantitative polymerase chain reaction (qPCR) was used to quantify *Acinetobacter* spp. 16S ribosomal ribonucleic acid (rRNA) copies in total deoxyribonucleic acid (DNA) extracts of biogas plant input and output samples. In addition, *Acinetobacter* spp. isolates cultured from input and output samples by different cultivation efforts (Schauss *et al.* 2015, 2016) were characterized in detail. The isolates were phylogenetically assigned based on 16S rRNA and *rpoB-gyrB* gene and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. Isolates identified as *A. baumannii* were examined by $bla_{\text{OXA-51}}$ typing and multilocus sequence typing (MLST) and comparative genome analysis. Isolates were further characterized with respect to several physiological properties as temperature-dependent growth and anaerobic condition survival tests as well as copper and antibiotic resistance patterns.

MATERIALS AND METHODS

Biogas plants and sampling

Input and output samples of 15 German biogas plants (BGP-1 to 15) located in the north of Hesse, near Aachen, Bremen, and in Bavaria near Munich were examined in 2012 and 2013. The studied biogas plants represented continuous stirred tank reactors (CSTRs) with wet fermentation processes (dry mass <15% w/v). Fourteen of the biogas plants were running under mesophilic (35–40°C) and one, BGP-12, under thermophilic conditions (50°C) (Table 1). More characteristics of the biogas plants, including volumes and retention times of the reception tank, the main and post-fermenters and the storage tank as well as the agitation technologies are given as supplementary material (Table S1, Supporting Information). All biogas plants, except BGP-7 and BGP-12, were fed with both, slurries and manure. BGP-12 was fed with manure only and BGP-7 with food leftovers. An overview of slurry and manure compounds and co-substrates is given in Table 1. Input samples investigated here contained slurry and manure components mixed in a ratio as added to the biogas digester (excluding bioenergy crops since the focus was set on resistant bacteria with potential exposure to veterinary antibiotics) (Table 1). Slurry and manure components were mixed respectively with a sterile stirrer in a sterile 50 L pot at the day of sampling. Output samples were obtained from half-day pre-mixed final storage tanks by randomly sampling a volume of 2 L every 10 min. During sampling, the storage tank was continuously mixed. The retention time was not considered because of identical sampling time points for input and output manure. Samples were taken in 250 mL sterile polyethylene (PE) bottles and stored immediately at 6°C, transported to the laboratory and processed on the same day for the cultivation-dependent approach. Samples used for molecular analysis were directly stored at −80°C until DNA extraction.

Total community DNA extraction from manure and anaerobic digestates

Total community DNA was extracted in triplicates from input and output materials of biogas plants using a phenol chloroform extraction method based on the method established by Carroll *et al.* (2012). For enzymatic cell lysis, samples were suspended in 750 μL lysis buffer [sodium chloride, 200 mM; ethylenediaminetetraacetic acid (EDTA), 100 mM; Tris base, 200 mM; pH 8.0] including lysozyme (20 mg mL^{-1}; AppliChem, Darmstadt, Germany), vortexed shortly and incubated at 37°C for 30 min. Subsequently, 80 μL of 10% (w/v) sodium dodecyl sulphate and 20 μL of proteinase K (50 mg mL^{-1} dissolved in water; Sigma-Aldrich, St. Lousi, Missouri, USA) were added. Samples were vortexed and incubated for 30 min at 60°C. To ensure the lysis of Gram-positive bacteria, samples were heated for additional 5 min at 95°C. Thereafter, samples were transferred into sterile 2-mL screw cap tubes containing ∼600 mg of 0.1 mm silica beads and four 2 mm glass beads (both Carl Roth, Karlsruhe, Germany). Screw cap tubes were autoclaved with beads before usage. Mechanical lysis of cells was performed by mixing screw cap tubes for 2 min into a mixer mill (MM2, Retsch, Haan, Germany). After centrifugation at 13 780 × *g* for 4 min at 4°C, the supernatant was transferred into sterile tubes containing 500 μL phenol/chloroform/isoamyl alcohol (Carl Roth). The tubes were vortexed for 15 s and centrifuged for 4 min at 4°C and 13 780 × *g* to dissolve humic acid substances; the upper phase containing nucleic acids was transferred to sterile tubes filled with 500 μL chloroform (Merck, Darmstadt, Germany), while the rest of the phenol and humic acid substances remained in the lower chloroform phase. For further purification of the nucleic acids, 1 mL of 100% ethanol and 45 μL of 0.3 M sodium acetate (Carl Roth) were added to the reaction tubes and incubated at −80°C for 1 h. Thereafter, the tubes were thawed and the nucleic acid precipitated by centrifugation for 4 min at 13 780 × *g*. The supernatant was discarded and tubes were placed into a vacuum concentrator (BaVaCo-M Mini-30, Bachofer, Reutlingen, Germany) for 10 min at room temperature to ensure complete liquid evaporation. The remaining DNA pellets were resuspended in 200 μL DNase and RNase free water (Carl Roth). Extracted nucleic acids were further purified using a QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instruction, and quantified by absorbance measurement using a NanoDrop spectrophotometer (NanoDrop 1000, Peqlab, Erlangen, Germany).

Quantification of *Acinetobacter* spp. 16S rRNA gene copies from total community DNA

Acinetobacter spp. 16S rRNA gene targets were quantified by qPCR using the *Acinetobacter* specific primer system Ac436f/Ac676r (Vanbroekhoven *et al.* 2004). In parallel, the total *Bacteria* 16S rRNA gene targets were quantified using the universal 16S rRNA gene sequence targeting primer system 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Lane 1991). All qPCR reactions were performed in a CFX96 Touch Real-time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA) with the SsoFast EvaGreen ® Supermix kit (Bio-Rad). Amplification was performed in a total volume of 10 μL including 5 μL of SsoFast EvaGreen ® Supermix, 1 μL of template DNA (5 ng μL^{-1}) and 0.2 μM of each primer. The final volume was adjusted with PCR water. The thermocycler program used for both primer systems was identical: 98°C for 2 min, 45 cycles at 98°C and 60°C for 5 s; a subsequent melting curve was generated by heating from 65°C to 95°C with 0.5°C increments at 5 s time. No primer dimer formation was obtained by melt curve analyses. All reactions were performed in technical duplicates. *Pseudomonas libanensis* DSM 17149^T was used as negative control for the *Acinetobacter* spp. specific PCR, and PCR grade water as no-template control for both primer systems. For quantification, a standard dilution series (10-fold dilution) of 16S rRNA gene fragment generated with the universal primers 27F/1492R (Lane 1991) from *Acinetobacter lwoffii* DSM 2403^T was used after purification with the QIAquick PCR purification Kit (Qiagen). The concentration of 16S rRNA gene targets was quantified based on DNA concentration (ng μL^{-1}) measured photometrically and the fragment length (in nt) as described by Kolb *et al.* (2003). The initial target copy number ranged from 10^1 to 10^9 16S rRNA gene targets per reaction and was plotted against Ct values to generate a standard curve ($r^2 > 0.98$; amplification efficiencies between 90 and 110%). The relative quantity of 16S rRNA genes of *Acinetobacter* in biogas plants was calculated by normalization to the bacterial 16S rRNA gene abundance to compensate for variance induced by amplification efficiency across samples. One-tailed pairwise *t*-tests were performed in SigmaPLOT 12.5 (Systat Software, San Jose, California, USA) to prove significant reduction of *Acinetobacter* 16S rRNA gene copies in digestates compared with manure.

Table 1. Characteristics of the studied German biogas plants: composition of input samples (slurry—manure), co-substrates applied to the fermentation and digestion layout of the studied BGPs. Modified based on Schauss *et al.* (2016). All plants are CSTRs.

Biogas plants	Input	Co-substrates (t per day)	Digestion process	Operation temperature
BGP-1	75% slurry (3:1 fattening pig, dairy cattle) 25% manure (6:0.5 cattle, laying hens)	8.5 corn 2 forage rye	two-stage, mesophilic	~40°C
BGP-2	50% slurry (3:1 fattening pig, dairy cattle) 50% manure (1:1:2 horse, chicken cattle)	8 corn 8 forage rye	two-stage, mesophilic	~40°C
BGP-3	15% slurry (10:1 cattle, fattening pig) 85% manure (4:1 cattle, horse)	2.2 corn 2 forage rye 0.4 grass silage 0.3 feed leftover	two-stage, mesophilic	~40°C
BGP-4	62% cattle slurry 37% cattle manure	10 corn 2.5 forage rye 1.5 grass silage	two-stage, mesophilic	~40°C
BGP-5	73% dairy cattle slurry 27% manure (2:1 cattle, fattening chicken)	9 corn 0.6 grass silage	one-stage, mesophilic	~40°C
BGP-6	83% slurry (1:1 dairy cattle to breeding sow) 17% dairy cattle manure	20 corn	one-stage, mesophilic	~40°C
BGP-7	2% slurry (cattle) 28% flotation tailings 70% food leftover	None	two-stage, mesophilic	~40°C; two-step hygienization between digester and storage tank step: 70°C
BGP-8	85% dairy cattle slurry 15% mother cow manure	6 corn 1 forage rye	one-stage, mesophilic	41.5°C
BGP-9	100% organic dairy cowslurry	None	one-stage, mesophilic	28–40°C
BGP-10	80% dairy cattle slurry 20% laying hens manure	9 corn	two-stage, mesophilic	~40°C
BGP-11	100% fattening bulls	36, crops are varying	two-stage, mesophilic	~40°C
BGP-12	100% chicken manure	25.5 corn	two lines, two-stage, thermophilic	~50°C
BGP-13	100% fattening pig slurry	8 corn 1 forage rye 1 grass silage	two-stage, mesophilic	41°C
BGP-14	53% fattening bull slurry 47% fattening bull manure horse manure	7.6 corn 1.5 grass silage	two-stage, mesophilic	~40°C
BGP-15	67% fattening bull slurry 33% turkey hen manure Additional turkey, hens manure sample	2.5 corn 1 forage rye 2 grass silage 1 shredded grain	two-stage, mesophilic	42°C

Cultivation of *Acinetobacter* spp. isolates

Acinetobacter spp. isolates were derived from different cultivation approaches including cultivation on R2A (Oxoid, Wesel, Germany), EMB (Merck) and CHROMagar ESBL (CHROMagar, Paris, France) agar plates. Bacteria were detached from 10 g manure and digestate samples using 0.2% filter-sterilized sodium pyrophosphate solution and serially diluted in 0.9% NaCl. All plates were incubated for 24 to 48 h at 37°C under aerobic conditions. For the cultivation of ESBL-producing bacteria, manure samples (0.1, 1 and 10 g) were additionally directly weighted into LB medium (Sigma-Aldrich) containing 1 mg L^{-1} cephalosporins [Cefotaxime ($C_{16}H_{16}N_5NaO_7S_2$) + Ceftazidime ($C_{22}H_{22}N_6O_7S$); Sigma-Aldrich]. After 24 h of incubation at 37°C, subsamples (10 μL) were streaked on CHROMagar ESBL (CHROMagar; Schauss *et al.* 2015). Carbapenem-resistant bacteria were cultured in parallel LB medium as enrichment broth containing 1 or 4 mg L^{-1} meropenem ($C_{17}H_{25}N_3O_5S$, Sigma-Aldrich) and CHROMagar KPC (CHROMagar) for selective streaking of enrichment cultures. Isolates were incubated for overnight at 37°C. Morphologically different colonies grown on agar plates were purified by transferring the single colony for multiple times. Fresh biomass of the isolates was suspended in newborn calf serum albumin (Invitrogen, Waltham, Massachusetts, USA) and stored at −20°C or −80°C for long-term preservation. For molecular characterization, cell lysates were generated from two loops of freshly grown biomass as described previously (Schauss *et al.* 2015).

Phylogenetic identification of *Acinetobacter* based on 16S rRNA gene and *rpoB/gyrB*

Partial 16S rRNA gene sequences of *Acinetobacter* isolates previously published by Schauss *et al.* (2015, 2016) were completed by sequencing the 3′ end of the 16 S rRNA gene using primer E786F (5′-GATTAGATACCCTGGTAG-3′; Colquhoun 1997). The 16S rRNA gene of remaining isolates was PCR-amplified using universal primers 27F/1492R as described by Glaeser *et al.* (2016) and sequenced using primer 27F and E786F by Sanger sequencing method (LGC Genomics, Berlin, Germany). Sequences were corrected and merged manually based on electropherograms by removing unclear 5′ and 3′ ends of the sequences in MEGA7 (Kumar, Stecher and Tamura 2016). Initial phylogenetic identification was performed using the EzBioCloud server (https://www.ezbiocloud.net/; Yoon *et al.* 2017) including 16S rRNA gene sequences of type strains of species with validated names (http://www.bacterio.net/-allnames.html). RNA polymerase β-subunit (*rpoB*) and the DNA gyrase subunit B (*gyrB*) genes were PCR amplified, and more comprehensive phylogenetic analyses were performed as described previously (Nemec *et al.* 2009; Krizova *et al.* 2014). The respective gene sequences of all isolates and all type strains and genomic species of *Acinetobacter* were aligned using ClustalW provided in MEGA7. The alignments of nucleotide sequences of the protein coding genes were performed according to the correct open reading frame (ORF). Phylogenetic trees were constructed in MEGA7 using the maximum-likelihood method based on the general time reversible model (Nei and Kumar 2000) and 100 re-samplings (bootstrap analysis). GenBank accession numbers of nucleotide sequences are MT138751–MT138756 (16S rRNA gene) and MT157622–MT157720 (*rpoB/gyrB*), respectively.

Differentiation of *Acinetobacter* isolates by genomic fingerprinting

All isolates were differentiated at the strain level by genomic DNA fingerprinting using repetitive DNA element PCR with $(GTG)_5$ primer (5′-GTGGTGGTGGTGGTG-3′; Versalovic, Schneider and Bruijn 1994), and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) with primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCA-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGC-3′) published by Versalovic, Koeuth and Lupski (1991). PCR amplification and agarose gel electrophoresis were performed as described by Glaeser *et al.* (2013, 2016). Cluster analysis of fingerprinting patterns was performed in Gel Compare II (Applied Maths, Sint-Martens-Latem, Belgium) with Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering based on a similarity matrix calculated using the Pearson correlation.

MALDI-TOF mass spectrometry-based identification

All isolates were further identified at the level of species by MALDI-TOF MS as described by Eisenberg *et al.* (2017). Briefly, bacterial isolates were selected from the culture plates and then subjected to steel-targets according to manufacturer's instruction (BrukerBiotyper, BrukerDaltonics, Bremen, Germany). Isolates were prepared using a direct transfer protocol provided by the manufacturer and analyzed on a Bruker Microflex LT system MALDI-TOF MS using Biotyper Version V3.3.1.0. The standard database used (DB 7311, BrukerDaltonics) comprised spectra from different *Acinetobacter* strains. The Compass software considers MALDI scores >2.3 and >2.0 as secure species and genus identification levels, respectively. The identification was repeated three times to verify the original findings.

$bla_{\text{OXA-51-like}}$ typing of *Acinetobacter* isolates

The presence of $bla_{\text{OXA-51-like}}$ genes was checked for all *Acinetobacter* spp. isolates. If the gene was detected, a PCR-based mapping of the location of the insertion sequence element (IS*Aba1*) upstream of $bla_{\text{OXA-51-like}}$ genes was performed using the primer pair ISA*ba*1F and OXA-51-likeR as described by Turton *et al.* (2006a). Complete sequence amplification and Sanger sequencing using forward and reverse primers was done according to Zander *et al.* (2012). All $bla_{\text{OXA-51}}$ variants were identified by BLAST (Basic Local Alignment Search Tool) analysis using the nucleotide BLAST platform. Novel OXA-51 variants were deposited in GenBank (accession numbers given in respective figures) and registered at Lahey database currently hosted at NCBI (https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamasedata-resources/). A maximum likelihood tree based on complete coding regions of the $bla_{\text{OXA-51-like}}$ gene was calculated in MEGA7 as described above for *rpoB/gyrB* analysis. Analysis included all of the international OXA-51 clonal lineages from Zander *et al.* (2012), variants of class D β-lactamases present in various *Acinetobacter* species mentioned by Périchon *et al.* (2014) and Wilharm *et al.* (2017).

Molecular typing for epidemiological and population-related study

MLST was performed with *A. baumannii* isolates using the Pasteur (Diancourt *et al.* 2010) and Oxford (Bartual *et al.* 2005) MLST schemes. Forward primers applied by Pasteur scheme were used for Sanger sequencing of all housekeeping gene sequences. Sequenced genes were corrected manually based on electropherograms. Sequences were deposited in GenBank with accession numbers MT157622–MT157720, respectively. Genes used in the Oxford scheme were retrieved from the genome sequences of *A. baumannii* isolates. Gene sequences were shortened to size required for MLST analysis using MEGA7 and sequence types (STs) were identified using both MLST database (http://pubmlst.org/abaumannii/). All STs represented in the Pasteur MLST database (https://pubmlst.org/bigsdb?db=pubmlst_abaumannii_pasteur_seqdef) were downloaded. The combined population structure of STs from our study and the Pasteur database was evaluated by generating minimum spanning tree (MST) from the Phyloviz (https://online.phyloviz.net/index; Francisco *et al.* 2012; Ribeiro-Gonçalves *et al.* 2016) using the goeBURST algorithm (Francisco *et al.* 2009).

Multilocus sequence analysis (MLSA)

MLSA based on concatenated sequences of all genes provided in Pasteur MLST scheme was performed with *A. baumannii* isolates, including avian strains (Wilharm *et al.* 2017), clinical strains and further type strains of species of the genus *Acinetobacter*. Nucleotide sequences were aligned according to the respective amino acid sequences and concatenated for analysis. A maximum likelihood tree was calculated as described above using MEGA7.

Whole genome sequencing, average nucleotide identity (ANI) and genome analysis of *A. baumannii* strains

Genomic DNA was extracted with the MasterPure DNA purification kit (Epicentre, Madison, Wisconsin, USA). Shotgun libraries were generated using the Nextera XT DNA sample preparation kit following the manufacturer's instructions. The whole genome shotgun libraries were sequenced using the dual index paired-end (v3, 2 × 300 bp) approach for the Illumina MiSeq

platform as recommended by the manufacturer (Illumina, San Diego, USA). Comparative genome analyses were performed in EDGAR (Blom *et al.* 2016). Genome sequences of the six *A. baumannii* isolates were deposited in NCBI under bioproject 'PRJEB35515', respectively. Average nucleotide identity (ANI) values were determined among the six *A. baumannii* isolates and to *A. baumannii* ATCC 19606^T and *A. calcoaceticus* DSM 30006^T (Table 2). IslandViewer 4 (Bertelli *et al.* 2017) for the island-like regions and IS Finder (Siguier *et al.* 2006) for insertion sequence (IS) elements were applied to search for resistance islands and IS elements.

Temperature-dependent growth and heavy metal ion tolerance tests

In order to determine the temperature range for growth, a spot assay technique described previously was applied (Aydogan *et al.* 2016). Plates were incubated at 4°C, 10°C, 15°C, 20°C, 25°C, 28°C, 30°C, 37°C, 45°C, 50°C and 55°C, respectively. Growth was monitored after two, three and seven days of incubation for all isolates. Copper tolerance was tested by preparing suspension of overnight cultured isolates in NaCl (0.9% w/v) adjusted to 0.5 McFarland turbidity. Serial dilutions up to 10^{-3} were performed in an empty sterile 96-well plate panel for total volume of 200 μL. Five microliters of each dilution was spotted on Müller-Hinton agar plates (Carl Roth) supplemented with various molar concentrations of $CuSO_4 \times 5H_2O$ (Merck) as described by Romero *et al.* (2017). The plates were incubated at 37°C aerobically and checked for growth after 24 h. The lowest copper concentration that suppressed growth was considered as MIC value.

Survival of *Acinetobacter* in anaerobic condition

All isolates were checked for survival or growth in anaerobic environment in controlled laboratory conditions. The survival of isolates under anaerobic condition was checked by taking isolates pre-grown (overnight aerobically at 25°C) on nutrient agar (Merck) plates, and exposing to the anaerobic conditions in Anaerocult A system (Merck) at the same temperature for seven days. Following anaerobic cultivation, a loop biomass was re-inoculated onto the fresh nutrient agar, and growth was checked after overnight aerobic incubation at 37°C. The ability of isolates to grow in anaerobic conditions was performed by direct exposure of streaked plates to the anaerobic conditions in Anaerocult A system at 25°C for seven days.

Antimicrobial susceptibility test of *Acinetobacter*

Antimicrobial susceptibility test for all isolates was performed with standard Micronaut-S test panel (Merlin, Bornheim-Hersel, Germany) including veterinary relevant antibiotics to determine minimum inhibitory concentrations (MICs in mg L^{-1}) using CLSI guidelines M100-S23 (Clinical and Laboratory Standards Institute 2012) as described previously (Schauss *et al.* 2015; Glaeser *et al.* 2016). *A. baumannii* isolates were additionally characterized using the MRGN Micronaut-S System (Merlin) as described by Müller *et al.* (2018). Classification into sensitive (S), intermediate (I) and resistant (R) against tested antibiotics was done according to EUCAST (http://www.eucast.org/clinical_breakpoints/) and CLSI databases of clinical breakpoints.

RESULTS

Presence of *Acinetobacter* spp. 16S rRNA gene targets in input and output samples of German biogas plants

The concentrations of *Acinetobacter* spp. specific 16S rRNA gene copies were in the range of 10^6 to 10^8 copies per g fresh weight (FW) in input and 10^5 to 10^6 in output materials. The concentration decreased between one and three orders of magnitude by comparing the input and output samples of individual biogas plants. For eight of the biogas plants the decrease was statistically significant (one-tailed pairwise *t*-test; $P < 0.01$ to $P < 0.05$; Fig. 1; Table S2, Supporting Information). In contrast to the concentration of *Acinetobacter* spp. 16S rRNA gene copies, the concentrations of 16S rRNA gene copies of total *Bacteria* were in the range of 10^{10} g^{-1} FW in input and output samples, respectively. No significant differences were observed (pairwise *t*-tests; $P > 0.05$).

Phylogenetic identification of *Acinetobacter* spp. cultured from manure and biogas plant digestates

A total of 17 *Acinetobacter* spp. isolates were cultured; 12 from input and 5 from output samples of various biogas plants (Table 2). Based on the phylogenetic analysis of nearly full-length 16S rRNA gene sequence eight isolates of input and output samples were placed into the ACB cluster, including two isolates with highest 16S rRNA gene sequence similarity to the type strain of *A. calcoaceticus* (>99.8%; both BGP-1) and six isolates with highest 16S rRNA gene sequence similarity to *A. baumannii* (>99.6%; two isolates from BGP-1, one each from BGP-5, BGP-6, BGP-12 and BGP-15) (Table 2). The other isolates of the input samples showed highest 16S rRNA gene sequence similarity to *A. towneri* (>98.7%; two isolates; both BGP-15), *A. beijerinckii* (99.6%; one isolate; BGP-5), *A. bereziniae* (99.5%; one isolate; BGP-6), *A. guillouiae* (98.7%; one isolate; BGP-5) and *A. indicus* (99.5%; one isolate; BGP-1) (Table 2). Other output isolates were next related to *A. lwoffii* (98.9% similarity; two isolates; both BGP-1) and *A. baumannii* but with a 16S rRNA gene sequence similarity of 97% (one isolate; BGP-1) and without clustering into the ACB cluster (Figure S1, Supporting Information).

MALDI-TOF data (Table 2) and phylogenetic analysis based on *rpoB/gyrB* sequences (Fig. 2) confirmed the phylogenetic assignment obtained by the 16S rRNA gene sequencing approach. Most of the isolates were identified by MALDI-TOF with log score hits >2.0 to the respective species mentioned above. There were three exceptions: isolates 794B1–12ER2A, next related to *A. indicus* (99.5%); 574B5–12EESBL, next related to *A. guillouiae* (98.7%); and KPC-SM-21, next related to *A. baumannii* (97.0%). Score values for those three isolates were too low for species assignment.

The phylogenetic tree calculated based on *rpoB/gryB* gene sequences confirmed the placement of the six *A. baumannii* isolates and the two *A calcoaceticus* isolates into the ACB cluster and other isolates next to the type strains of the abovementioned species (Fig. 2). Isolate KPC-SM-21 (97% 16S rRNA gene similarity to *A. baumannii*) clustered with high bootstrap support next to *A. gerneri* DSM 14967^T. As expected, the six isolates assigned as *A. baumannii* clustered also with *A. baumannii* ATCC 19606^T in MLSA approach based on seven different housekeeping genes used in the Pasteur MLST scheme (Figure S2, Supporting Information). Comparative genomic fingerprint analyses including BOX- and $(GTG)_5$-PCRs showed distinct genomic fingerprint types for 15 of the isolates, which indicated that they represented different strains (Fig. 3). Only two isolates, 551B1–

Table 2. Overview of isolates and comparison of different investigated German biogas plants. MALDI-TOF score values for species and genus level identification are log(score) ≥2.0 and log(score) 1.7–2.0, respectively. A log score below 1.7 did not enable a genus or species assignment.

	Isolates	Biogas plant	Phylogenetic calculations 16S rRNA gene-based phylogenetic identification	MALDI-TOF MS Organism (best match)	Log score	(ANI) % *A. baumannii* ATCC 19606^T vs BGPs strain
1	**KPC-SM-17a**	BGP-1 (I)	99.8% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.22	97.4%
2	**552B1–12EESBL**	BGP-1 (I)	99.9% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.33	97.57%
3	**571B5–12EESBL**	BGP-5 (I)	99.8% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.29	97.64%
4	**901B6–12EESBL**	BGP-6 (I)	99.7% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.36	97.59%
5	**KPC-SM-125**	BGP-15 (O)	99.8% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.33	97.57%
6	**945B12–12AESBL**	BGP-12 (O)	99.8% *A. baumannii* ATCC 19606^T	*A. baumannii*	2.31	97.6%
7	551B1–12EESBL	BGP-1 (I)	100% *A. calcoaceticus* DSM 30006^T	*A. calcoaceticus*	2.53	n.d.
8	553B1–12EESBL	BGP-1 (I)	99.9% *A. calcoaceticus* DSM 30006^T	*A. calcoaceticus*	2.62	n.d.
9	794B1–12ER2A	BGP-1 (I)	99.5% *A. indicus* CIP 110367^T	n.d.	1.59	n.d.
10	574B5–12EESBL	BGP-5 (I)	98.7% *A. guillouiae* CIP 63.46^T	n.d.	1.62	n.d.
11	734B5–12EEMB	BGP-5 (I)	99.6% *A. beijerinckii* CIP 110307^T	*Acinetobacter* sp.	2.36	n.d.
12	815B5–12ER2A	BGP-5 (I)	99.3% *A. towneri* DSM 14962^T	*A. towneri*	2.39	n.d.
13	902B6–12EESBL	BGP-6 (I)	99.5% *A. bereziniae* LMG 1003^T	*A. bereziniae*	2.34	n.d.
14	KPC-SM-69	BGP-15 (I)	98.8% *A. towneri* DSM 14962^T	*A. towneri*	1.87	n.d.
15	KPC-SM-21	BGP-1 (O)	97% *A. baumannii* ATCC 19606^T	n.d.	1.56	n.d.
16	KPC-SM-24	BGP-01 (O)	98.9% *A. lwoffii* NCTC 5866^T	*A. lwoffii*	1.75	n.d.
17	KPC-SM-26	BGP-01 (O)	98.9% *A. lwoffii* NCTC 5866^T	*A. lwoffii*	1.76	n.d.

O: output sample; I: input sample; KPC: CHROMagar KPC (CHROMagar, Paris, France); EMB: eosin-methylene blue (Merck); R2A: Oxoid. Isolates with 16S rRNA gene sequence similarity to *A. baumannii* ATCC 19606^T were represented in bold. 'n.d.' = not determined by MALDI-TOF MS and ANI.

12EESBL and 553B1–12EESBL, which were isolated from the same sample, shared identical fingerprint patterns indicating clonality. The genetically different *A. baumannii*-related isolates were considered subsequently as strains.

Genome sequence-based characterization of *A. baumannii* strains

The six *A. baumannii* strains were further investigated by genome sequencing. The obtained draft genomes had a size ranging from 3.08 to 4.16 Mbp. Details of all genomes are provided in NCBI under bio project accession number PRJEB35515. Pairwise ANI values among the genomes of the six strains and *A. baumannii* ATCC 19606^T were in the range of 97.28–97.67% (Table 2). Cluster analyses based on pairwise ANI values showed a single unique cluster determined for all *A. baumannii* strains of this study and respective type strains of *A. baumannii* (Figure S3, Supporting Information). All values were above the species boundary of 95–96% (Richter and Rosselló-Móra 2009), which confirmed the assignment of the six strains to the species *A. baumannii*.

bla_{OXA-51} typing of *A. baumannii* strains

All six *A. baumannii* strains contained a $bla_{OXA-51-like}$ gene; however, no PCR products instead were obtained by bla_{OXA-51} PCR screening for remaining *Acinetobacter* isolates. Protein BLAST of the complete $bla_{OXA-51-like}$ gene sequences of the *A. baumannii* strains was performed to identify their OXA-51-like type. Four known OXA-51-like types, OXA-104, OXA-106, OXA-343 and OXA-91, and two novel variants (OXA-909 and OXA-910) were determined. Phylogenetic analyses based on $bla_{OXA-51-like}$ gene sequences showed a clustering of the *A. baumannii* strains from manure and digestates with avian and clinical strains of *A. baumannii* (Fig. 4). The lack of insertion sequence element IS*Aba1* upstream of $bla_{OXA-51-like}$ genes in all *A. baumannii* strains was confirmed by PCR and comparative genome analysis (Figure S4, Supporting Information).

MLST of *A. baumannii* strains

MLST was applied to study the six *A. baumannii* strains in an epidemiological context. Based on the applied Pasteur MLST scheme five strains represented novel ST types (ST1442^P, ST1443^P, ST1444^P, ST1445^P and ST1446^P) and one strain represented previously known ST46^P (Fig. 5; Table S7, Supporting Information). However, four strains were assigned as novel STs and two strains were determined as ST1210^{Ox} and ST1557^{Ox} based on Oxford scheme, respectively (Table S7, Supporting Information). An MST calculated with the goeBURST algorithm (Francisco *et al.* 2009) showed the placement of the six strains among the STs provided in the Pasteur MLST database scheme (both clinical and extra-clinical origin), and those obtained from wild and livestock avian samples (Wilharm *et al.* 2017) and bovine sources (Klotz *et al.* 2019).

Heavy metal resistance, antibiotic resistance and virulence genes

Genome sequences of all six *A. baumannii* strains and *A. baumannii* ATCC 19606^T (ACQB00000000) were compared in EDGAR, which confirmed the presence of copper-related efflux pump genes (*copA* and *copB*) in all strains studied (Figure S5, Supporting Information). Similarly, genome analysis revealed the presence of AmpC ß-lactamases (Class C type) and multiple intrinsic resistance-nodulation-cell division (RND) and multidrug and toxic compound extrusion (MATE) type efflux genes, including the chloramphenicol resistance gene *craA* and the fosfomycin resistance efflux gene *abaF* in the genome of all six strains (for details, see Fig. 6; Table S5, Supporting Information), and all six strains lacked insertion sequence and resistance islands. In all strains the transposition of IS*Aba1* into the *adeS* gene was absent. Five out of six strains (Table S5 and Figure S8, Supporting

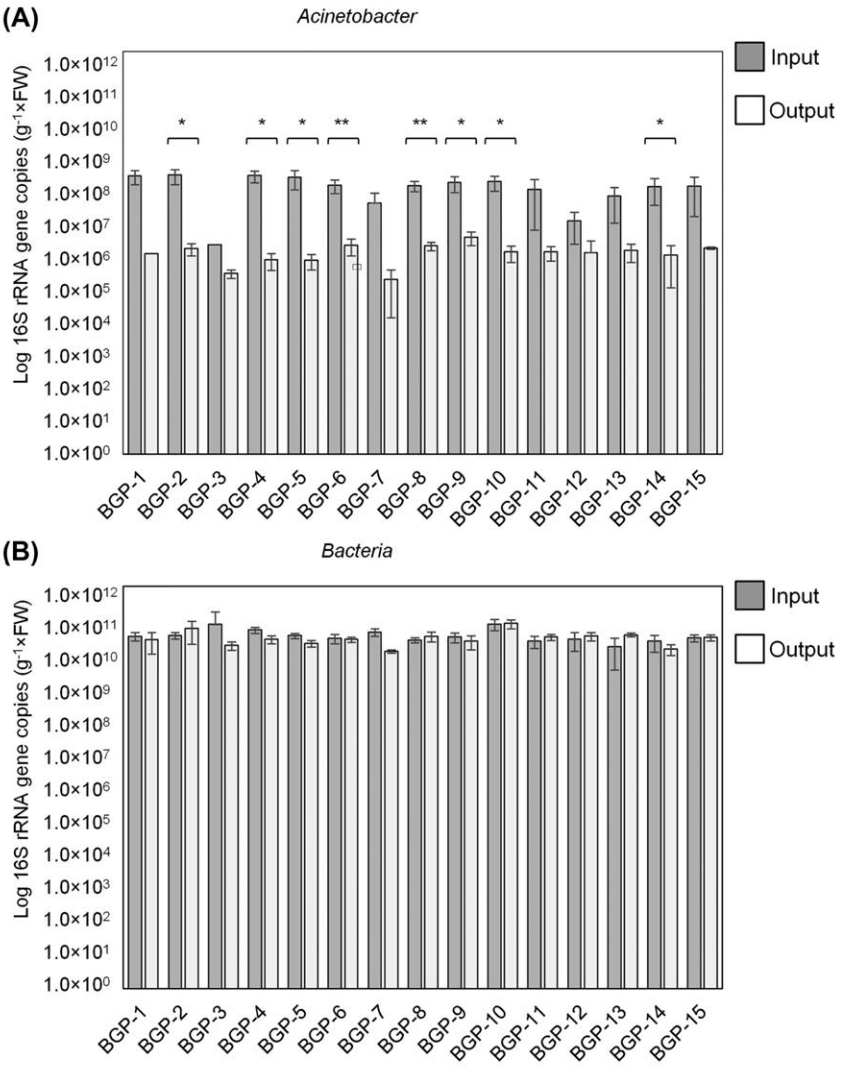


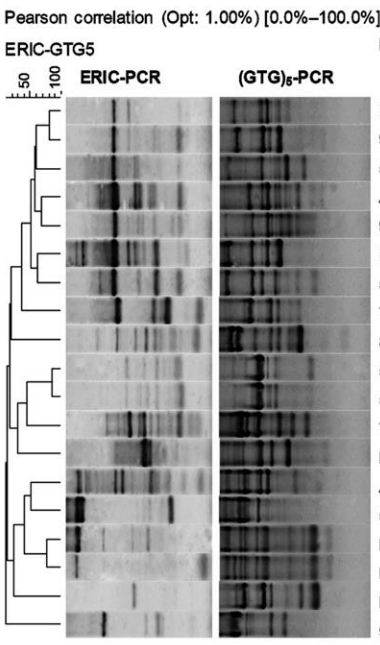
Figure 1. Concentrations of *Acinetobacter* **(A)** and *Bacteria* **(B)** 16S rRNA gene copies in input and output material of 15 German biogas plants. Mean values and standard deviation of qPCR measurements of three independent extracted total community DNA samples were shown. Asterisks indicated significant difference among input and output samples determined per biogas plant (*$P < 0.05$; **$P < 0.01$; pairwise *t*-test).

Information) in our study lacked the *adeC* gene. In all six strains, the *comM* gene was complete and not interrupted by a resistance island. Moreover, genome analysis also showed the presence of genes that code for most virulence factors like type II and type VI secretion systems, outer membrane protein A (OmpA), protein tyrosine kinase, putative polysaccharide export outer membrane protein (EpsA), iron starvation-related protein (Fe/S protein) and type I chaperone usher pili (Csu pili) required by this bacterium to thrive in both biotic and abiotic environments (Fig. 6; Figure S6 and Table S6, Supporting Information); however, all virulence-related genes were located in the core genome. In addition genes involved in processing of polyphosphates were determined in all *A. baumannii* strains. Among those, genes coding for AMP phosphotransferase and adenylate kinase were determined. Locus tag numbers are provided in Figure S7 (Supporting Information).

Temperature-dependent growth and survival of *Acinetobacter* in anaerobic condition

All *Acinetobacter* spp. isolates grew well between 30 and 37°C. The growth of some isolates was slightly reduced between 20–28°C, but growth of all isolates was weak at lower tested temperature (4–15°C). The highest growth temperature for most of the *Acinetobacter* spp. isolates from both input and output samples was 37°C. The exceptions were the six *A. baumannii* strains that still grew at 45 and 50°C but not at 55°C and the strain next related to *A. baumannii*, KPC-SM-21 (97.3% 16S rRNA gene sequence similarity) did also grow at 45°C but not at 50°C and above. The type strain of *A. baumannii* also grew at 45°C, but not at 50°C and above (Figure S9, Supporting Information). All *Acinetobacter* isolates were unable to grow under anaerobic conditions. However, all isolates were able to survive in anaerobic conditions on nutri-

Figure 2. Maximum likelihood tree based on concatenated nucleotide sequence of *rpoB-gyrB*, where *rpoB* reperesented the Z1 and Z2 regions described by Nemec *et al.* (2009). GenBank accession numbers were given in parentheses. Bootstrap values >70% (100 resamplings) were indicated. Bar: 0.01 substitutions per nucleotide position.



Isolate/Type strain	Phyl. identification	BGP-(I/O)	$bla_{OXA-51-like}$	Highest growth temp.	$CuSO_4$ (MIC)	Effux/β-lactamase genes
KPC-SM-17a	99.8% *A. baumannii*	1-(I)	100% OXA-91	50°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
901B6-12EESBL	99.7% *A. baumannii*	6-(I)	99% OXA-377	50°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
552B1-12EESBL	99.9% *A. baumannii*	1-(I)	100% OXA-343	50°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
A. baumannii ATCC 19606^T			+	45°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
945B12-12AESBL	99.8% *A. baumannii*	12-(O)	100% OXA-106	50°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
KPC-SM-125	99.8% *A. baumannii*	15-(O)	99% OXA-69	50°C	8 mM	*adeABC, adeRS, adeIJK, abaF abeM, craA/ampC*
571B5-12EESBL	99.8% *A. baumannii*	5-(I)	100% OXA-104	50°C	8 mM	*adeAB, adeRS, adeIJK, abaF abeM, craA/ampC*
734B5-12EEMB	99.6% *A. beijerinckii*	5-(I)	-	37°C	4 mM	n.d.
815B5-12ER2A	99.3% *A. towneri*	5-(I)	-	37°C	4 mM	n.d.
551B1-12EESBL	100% *A. calcoaceticus*	1-(I)	-	37°C	8 mM	n.d.
553B1-12EESBL	99.9% *A. calcoaceticus*	1-(I)	-	37°C	8 mM	n.d.
794B1-12ER2A	99.5% *A. indicus*	1-(I)	-	37°C	4 mM	n.d.
KPC-SM-21	97% *A. baumannii*	1-(O)	-	45°C	8 mM	n.d.
A.lwoffi NCTC 2403^T			n.d.	n.d.	n.d.	n.d.
574B5-12EESBL	98.7% *A. guillouiae*	5-(I)	-	37°C	8 mM	n.d.
KPC-SM-24	98.9% *A. lwoffii*	1-(O)	-	37°C	4 mM	n.d.
KPC-SM-26	98.9% *A. lwoffii*	1-(O)	-	37°C	4 mM	n.d.
KPC-SM-69	98.8% *A. towneri*	15-(I)	-	37°C	4 mM	n.d.
902B6-12EESBL	99.5% *A. bereziniae*	6-(I)	-	37°C	8 mM	n.d.

Figure 3. Cluster analysis of *Acinetobacter* spp. isolates based on genomic fingerprints generated by ERIC-PCR and $(GTG)_5$-PCR. Cluster analysis was performed in GelCompare II using the UPGMA clustering method based on the Pearson correlation. BGP: biogas plant; I: input; O: output sample. Intrinsic $bla_{OXA-51-like}$ genes variants; '+': positive; '−': negative. 'n.d.': not determined.

ent agar plates for a week at 25°C, and thereafter grew well in aerobic conditions at 37°C (data not shown).

Copper tolerance of *Acinetobacter* spp. isolates

All *Acinetobacter* spp. isolates were tested with respect to their growth in the presence of copper. The six *A. baumannii* strains including the type strain of *A. baumannii* and two other isolates grew well in the presence of 4 mM copper and three other isolates showed reduced growth at that copper concentration (Figure S10, Supporting Information). None of the isolates grew in the presence of 8 mM copper and at higher copper concentrations. Besides the six *A. baumannii* strains, the two isolates assigned to *A. calcoaceticus*, 553B1–12EESBL and 551B1–12EESBL, and the isolate next related to *A. baumannii*, KPC-SM-21, were among the isolates that grew in the presence of 4 mM copper.

Antimicrobial susceptibility patterns of *Acinetobacter* spp. isolates

All isolates including *A. baumannii* strains were susceptible to most of the tested antibiotics like cephalosporins (cefotaxime and ceftazidime), carbapenems (meropenem and imipenem), piperacillin, quinolones (levofloxacin and ciprofloxacin), tetracycline, trimethoprim/sulfamethoxazole and polymyxin E (colistin). Data of antibiotic susceptibility patterns are provided in Tables S3 and S4 (Supporting Information).

DISCUSSION

This study showed a high abundance of 16S rRNA gene copies of *Acinetobacter* spp. in input and output samples of German biogas plants. Although the copy number was significantly reduced in output materials of several biogas plants, the concentration was still in a high range. Several different *Acinetobacter* spp. were isolated in the presence of cephalosporins and carbapenems. Isolates were next related to *A. baumannii*, *A. calcoaceticus*, *A. towneri*, *A. beijerinckii*, *A. bereziniae*, *A. defluvii*, *A. indicus* and *A. lwoffii* (Table 2). The detailed characterization of the six strains assigned to the species *A. baumannii* further illustrated a high diversity of *A. baumannii* released via manure and pretreated digestate from biogas plants into the environment.

Even though the genus *Acinetobacter* is generally regarded as obligate aerobic, its members have been known to occur in different anaerobic or oxygen-limited environments, including anaerobic digesters (Supaphol *et al.* 2011; Baek, Kim and Lee 2014; Jo *et al.* 2015). Recently Higgins *et al.* (2018) reported that *A. baumannii* survived the activated anaerobic mesophilic sludge digestion in wastewater treatment plants, but were ultimately destroyed in alkaline lime-treated stabilized sludge. The authors illustrated in lab scale tests that *A. baumannii* were not able to grow under anaerobic conditions but survived an incubation period of four weeks under the same conditions. The authors pointed out that anaerobic treatment also enables the survival and dissemination of this nosocomial obligate aerobic pathogen. Here we confirmed that finding by the determination of *Acinetobacter* spp. in digestates of anaerobic biogas fermenters. In agreement with findings of Higgins *et al.* (2018), all *Acinetobacter* isolates (including *A. baumannii* strains) of this study were able to survive in anaerobic laboratory test conditions. So far, it is still unclear how *Acinetobacter* can survive under anaerobic conditions. Earlier studies have shown that *Acinetobacter* spp. were able to accumulate efficiently intracellular polyphosphates, and thereby contributing to a minor extent to the phosphate elimination in sewage treatment plants (Fuhs and Chen 1975; Deinema *et al.* 1980; Deinema, Van Loosdrecht and Scholten 1985; Wentzel et al. 1986, 1991; Stephenson 1987; Bark *et al.* 1992). Van Groenestijn *et al.* (1987) reported that the accumulated polyphosphates in *Acinetobacter* cells act as a phosphorus reserve and might be used as energy source by enzymatic processing of the polyphosphates via combined action of polyphosphates: AMP phosphotransferase and an adenylate kinase. Comparative genome analyses revealed the presence of genes that code for these enzymes in all six strain of *A. baumannii* cultured in this study (Figure S7, Supporting Information). The described biological process can be an explanation

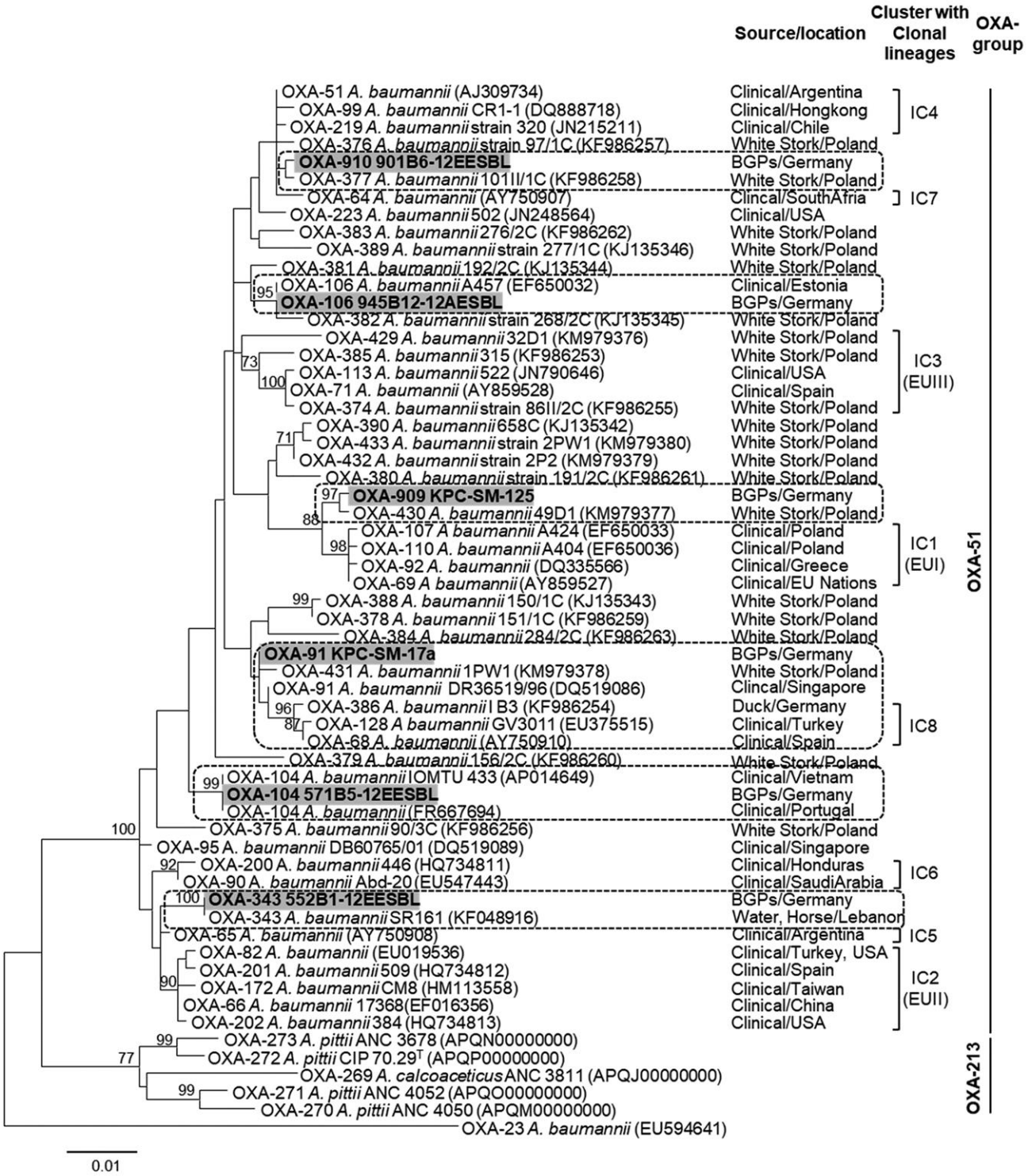


Figure 4. Correlation of complete nucleotide sequences of $bla_{OXA\text{-}51\text{-}like}$ genes from this study with international clonal lineages, avian and clinical strains of *A. baumannii*, calculated by maximum likelihood method. GenBank accession numbers were represented in parentheses. Representatives of $bla_{OXA\text{-}213}$ and $bla_{OXA\text{-}23}$ were used as outgroup to root the tree. Bootstrap values >70% (100 re-samplings) were shown. Strains related to this study were given in bold font. International clonal lineages: IC; European Union clonal lineages: EU.

for the survival of aerobic organism in anaerobic biogas plant or anaerobic sludge treatment in waste water treatment plants, because the polyphosphate reserve in *Acinetobacter* cells can be vital under anaerobic environment conditions when these strict aerobic organisms have no other options to generate energy (Kortstee *et al.* 1994).

Survival and transfer of potentially pathogenic culturable antibiotic-resistant bacteria and antibiotic resistance genes in

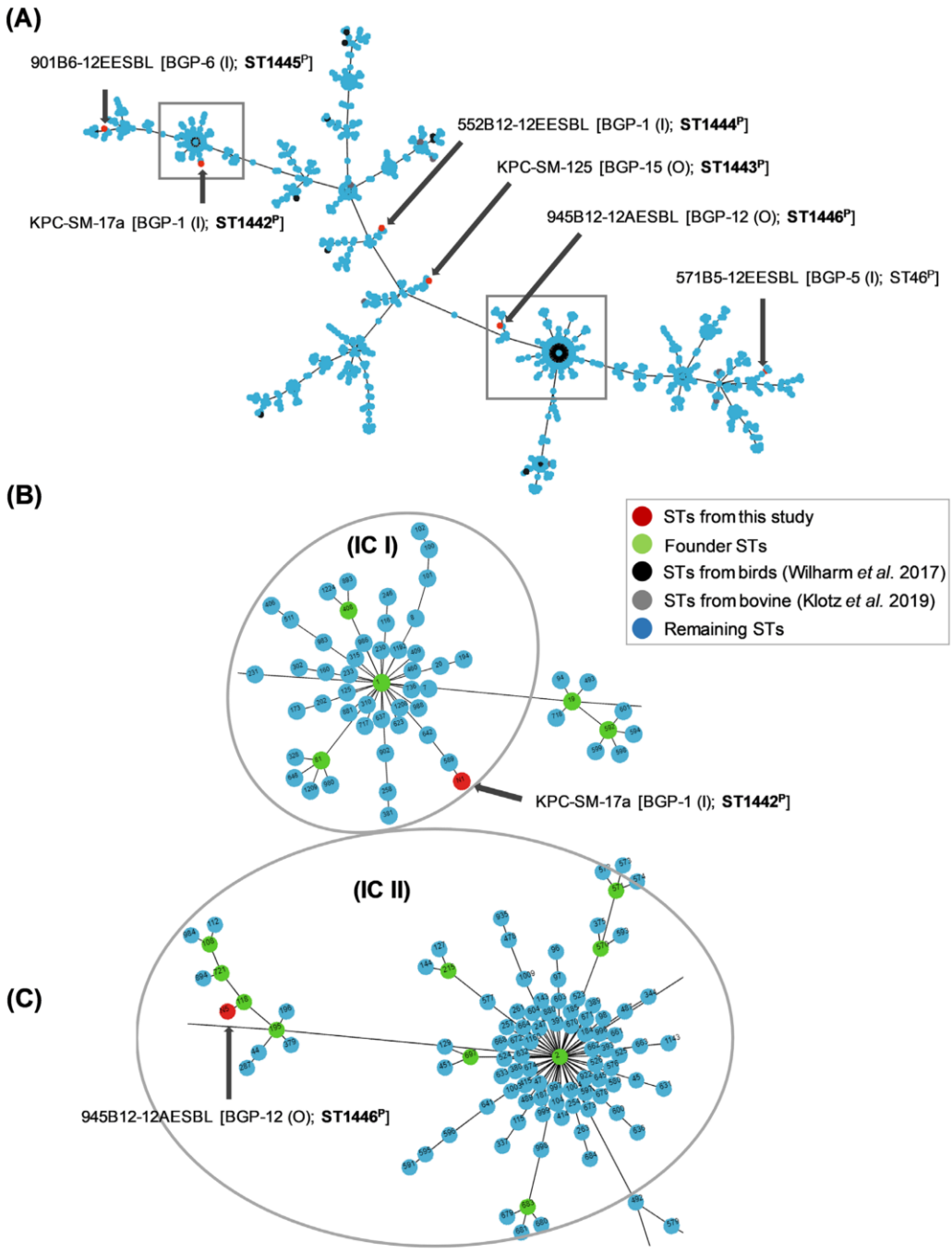


Figure 5. (A) MST generated by goeBURST algorithm using MLST profile data from Pasteur scheme, avian (Wilharm *et al.* 2017) and bovine (Klotz *et al.* 2019) related STs and those obtained from this study. Black and gray indicated avian and bovine STs. Partial snapshot of MST showing population structure of *A. baumannii* strains in **(B)** international clonal complex 1 (IC-1) and **(C)** international clonal complex 2 (IC-2) with STs of this study. Green and blue circles resembled founder and remaining STs from Pasteur database (both clinical and extra-clinical origin). The black arrows point to STs related to this study, which were labeled with red circles. Isolation source and STs were given in parentheses. Novel STs were given in bold font.

anaerobic treatment processes have been reported in few previous studies (Beneragama *et al.* 2013; Resende *et al.* 2014; Schauss *et al.* 2015; Glaeser *et al.* 2016; 2016; Wolters *et al.* 2016). In a recent study, Song *et al.* (2017) provided insights into the dissemination of antibiotic resistance genes in anaerobic digestion systems, and showed that *Acinetobacter* and other genera are potential hosts of erythromycin resistance mediating methylase (*ermB*), sulfonamide resistance due to dihydropteroate synthase (*sul1*) and trimethoprim resistance by class 1 integron-borne (*dfrA7*) genes. Intriguingly, there exist several factors that impact the reduction of pathogens in anaerobic digestion, for instance, temperature (Martin, Bostian and Stern 1990), moisture content (Russ and Yanko 1981), biosolids (Sidhu and Toze 2009), efficient mixing and organic matter stabilization (Smith *et al.* 2005) and composition of indigenous microorganisms (Pietronave *et al.* 2004).

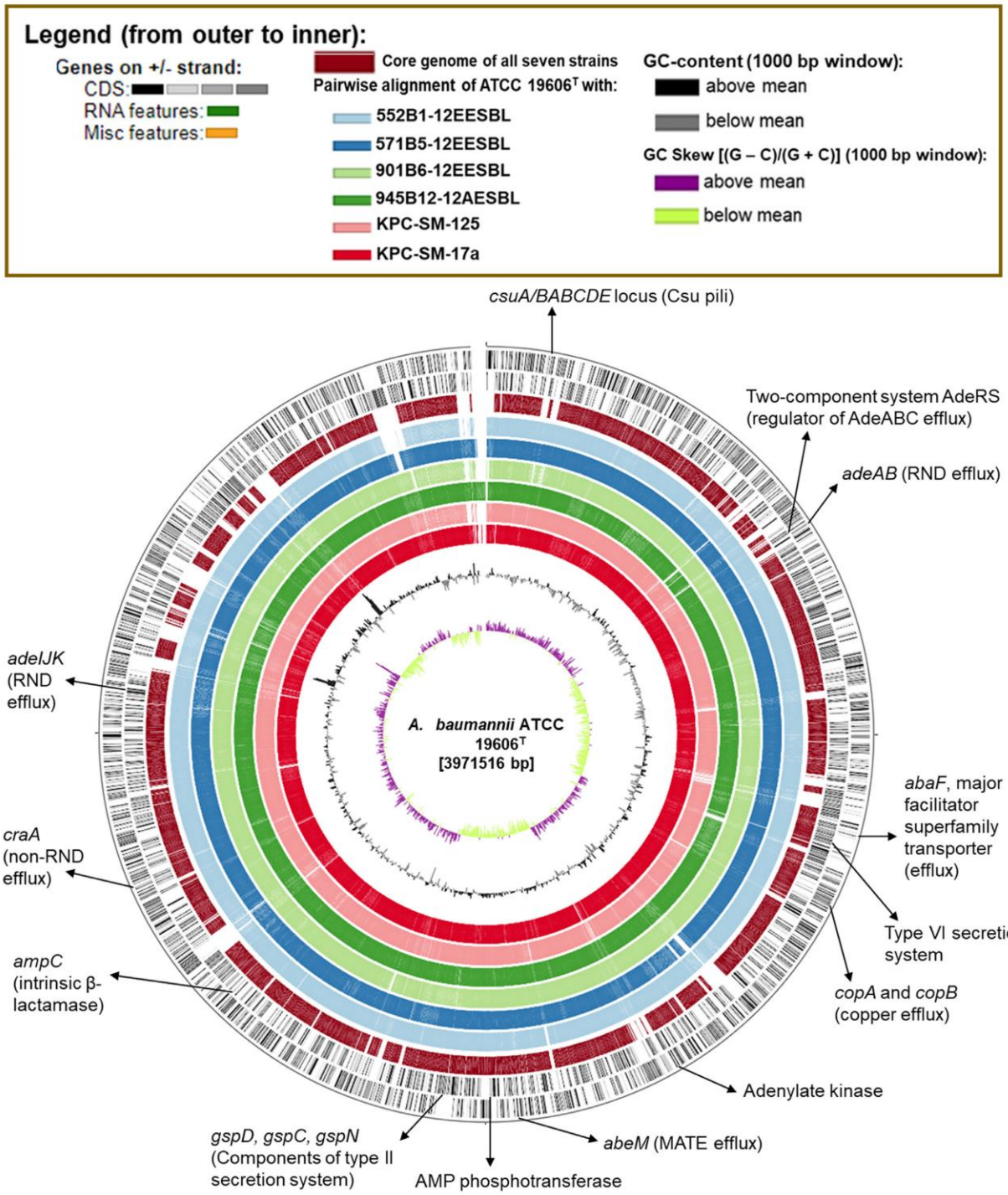


Figure 6. Circular plot of whole genome of six strains of *A. baumannii* isolated from German biogas plant. The whole genome (size in the middle of plot) of *A. baumannii* ATCC 19606^T was used as reference represented by two outer circles. Circular plot was generated with BioCircos (Cui *et al.* 2016) implemented in EDGAR (Blom *et al.* 2016). All labeled genes were found to be intrinsic to the genome of respective strains.

In this study, six strains of *A. baumannii* were identified, of which two strains originated from effluent samples of two different biogas plants (Table 2). To the best of our knowledge, there are only few more reports regarding the presence of *A. baumannii* in effluent source after anaerobic digestion processes (Resende *et al.* 2014; Schauss *et al.* 2015), and *A. baumannii* isolates cultured from the anaerobic digesters were not studied in detail yet.

In the Oxford scheme based MLST of *A. baumannii* strains, two previously known and four novel STs were identified. Using the Pasteur MLST, one previously known and five novel STs were determined. The known STs did not represent the major STs of international clonal complex (IC) 1 and 2 (Pasteur based) or CC109^{Ox} and CC92^{Ox} (Oxford based) responsible for outbreak of global epidemiology (Zarrilli *et al.* 2013). Based on the suggestion

of Gaiarsa *et al.* (2019), STs from Pasteur scheme were used to generate combined population structures through MST calculation, since the Pasteur scheme provides higher precision for discriminating strains among clonal groups and is less influenced by homologous recombination.

The antibiotic susceptibility test showed that *A. baumannii* strains were still susceptible to third- and fifth-generation cephalosporins and their combinations (cefotaxime, ceftaxidime, ceftolozan), cabapenems and colistin (Table S4, Supporting Information). However, with the exception to the fourth-generation cephalosporins (cefquinome) with and without clavulanic acid, amoxicillin, enrofloxacin, tetracycline and trimethoprim/sulfamethoxazole, *A. baumannii* strains under this study had relatively higher MIC values compared with other non-*A. baumannii* isolates for tested antibiotics (Table S3, Supporting Information). The genomes of *A. baumannii* strains contained intrinsic class C type β-lactamase and multiple efflux pump-related genes (Fig. 6; Table S5, Supporting Information). The higher MIC values against oxacillin, the third-generation cephalosporin ceftiofur (with or without clavulanic acid), and florfenicol (Table S3, Supporting Information) and against temocillin, fosfomycin and chloramphenicol (Table S4, Supporting Information) could be due to the basal-level expression of RND type efflux genes like *adeABC* (regulated by two-component regulatory operon, *adeR-adeS*) and *adeIJK*, multidrug and toxic compound extrusion (MATE) type efflux gene, e.g. multidrug efflux *abeM*, chloramphenicol resistance gene *craA* (reviewed by Coyne, Courvalin and Périchon 2011), fosfomycin resistance efflux gene *abaF* (Sharma *et al.* 2017) and *ampC* cephalosporinase (Corvec *et al.* 2003). All these genes were not located in resistance islands but rather were intrinsic to the core genomes (Table S5, Supporting Information). As expected, all *A. baumannii* strains in our study lacked point mutations in conserved regions of the AdeRS system that were reported by Yoon, Courvalin and Grillot-Courvalin (2013) mostly among multidrug-resistant clinical isolates of *A. baumannii*. Transposition of ISA*ba1* into the *adeS* gene was linked with overexpression of this efflux system in clinical isolates (Ruzin, Keeney and Bradford 2007); however, this gene was intact in all *A. baumannii* strains of our study (Figure S8, Supporting information). Five out of six strains (Table S5 and Figure S8, Supporting Information) in our study lacked *the adeC* gene, which has been considered as non-essential for the multidrug-resistant phenotype conferred by the efflux pump based on earlier studies (Marchand *et al.* 2004; Nemec *et al.* 2007). Insertion of IS element upstream was reported for hyperexpression of *ampC* gene in clinical *A. baumannii* (Corvec *et al.* 2003); however, all six strains lacked insertion at the upstream of *ampC* gene. Although *A. baumannii* is intrinsically resistant to these veterinary (florfenicol and ceftiofur) and clinical (temocillin, fosfomycin and chloramphenicol) antibiotics (Coyne, Courvalin and Périchon 2011), there are some reports on the use of fosfomycin in combination with other drugs, such as colistin, minocycline and polymyxin against *Acinetobacter* (Zhang *et al.* 2013b;Sirijatuphat and Thamlikitkul 2014).

Similar to our study, *A. baumannii* derived from dairy cows, beef cattle and calves of German livestock (Klotz *et al.* 2019) and avian sources (Wilharm *et al.* 2017) carried much less acquired resistances compared with strains derived from companion animals and human activities influenced sources like wastewater that harbored acquired $bla_{\text{OXA-23, -72}}$ and bla_{TEM} genes (Ewers *et al.* 2017; Higgins *et al.* 2018). Similarly, the comparative genome analysis showed the presence of genes that code for most virulence factors like type II and VI secretion systems, OmpA, EpsA, tyrosine kinase, Fe/S protein and Csu pili, which are required by this bacterium to thrive in both biotic and abiotic environments (Weber *et al.* 2017; Harding, Hennon and Feldman 2018).

Apart from clinical settings, *A. baumannii* has been described as a soil organism, however, without the support of enough specific references (Fournier *et al.* 2006). The presence of *A. baumannii* strains outside clinical settings is still an issue of controversial debate. Houang *et al.* (2001), Vangnai and Petchkroh (2007) and Hamouda *et al.* (2011) have already reported *A. baumannii* strains among samples collected from vegetables, fish, meat, soil and agricultural fields. Most recently, Lupo *et al.* (2014), Rafei *et al.* (2015), Wilharm *et al.* (2017), Kittinger *et al.* (2018) and Klotz *et al.* (2019) successfully isolated *A. baumannii* from water, soil, farm animals and their products and from avian samples including wild birds, suggesting that animals and birds might represent reservoirs for *A. baumannii* with the potential of dissemination of new emerging carbapenemase, such as $bla_{\text{OXA-143}}$ to humans. The influent manure (Table 1) used in biogas plants mentioned in this study was mostly originating from animal husbandries and poultry farms. Therefore, our results are in congruence with the findings of Wilharm *et al.* (2017) and Klotz *et al.* (2019) suggesting that livestock and birds could be secondary habitats of this bacterium. In contrast to several other pathogens, the main colonization site of *A. baumannii* in animals is still unknown. Klotz *et al.* (2019) cultured *A. baumannii* mainly from nasal swabs but also from feces samples and determined ST-types, which seemed to be able to colonize cattle and humans as well. Similarly, detailed analysis of virulence proteins of *A. baumannii* strains isolated from diseased chicken and isolation of this bacterium carrying the highly problematic $bla_{\text{NDM-1}}$ resistance gene from a lung sample of a pig with pneumonia and sepsis might point toward a potential zoonotic role for multidrug-resistant *A. baumannii* (Zhang *et al.* 2013a; Liu *et al.* 2016).

All *A. baumannii* strains of our study harbored the $bla_{\text{OXA-51-like}}$ gene, which is considered an intrinsic class D β-lactamase (Turton *et al.* 2006a). This gene is naturally acting as a weak β-lactamase, nevertheless, the β-lactam hydrolyzing activity can be increased by upstream insertion of ISA*ba1* (Turton *et al.* 2006a). Since *A. baumannii* is one of the members of ACB complex, its identification is equally challenging and time consuming although multiple techniques have been developed (Tjernberg and Ursing 1989; Gerner-Smidt, Tjernberg and Ursing 1991; Peleg, Seifert and Paterson 2008; Nemec *et al.* 2011). Therefore, our findings support the notion that amplification of intrinsic $bla_{\text{OXA-51-like}}$ gene is recommended for preliminary identification of *A. baumannii* from ACB complex as suggested by Turton *et al.* (2006b), because this gene has been rarely reported in non-*A. baumannii* complex species (Lee *et al.* 2009). Besides the members of ACB complex, *A. lwoffii* being ubiquitous in nature and considered to belong to normal microbiota known to inhabit human skin is an emerging opportunistic pathogen within the genus *Acinetobacter* (Bouvet and Grimont 1986; Turton *et al.* 2010). Several reports of infections and clinical manifestation of this bacterium have been published (Rathinavelu, Zavros and Merchant 2003; Tega *et al.* 2007; Hu *et al.* 2011; Singh *et al.* 2016). The presence of strains of opportunistic pathogens *A. baumannii* and *A. lwoffii* in output sources of biogas plants may pose environmental and epidemiological risks, because multidrug-resistant strains of these bacteria are known to spread and take up multiple antibiotic resistance genes in clinical and environmental settings mostly via horizontal gene transfer (Fournier *et al.* 2006; Visca, Seifert and Towner 2011; Tanner *et al.* 2017).

It was also proven that unleashed pathogenic bacteria from anaerobic digestion could survive in the field sites after application of digestate as manure (Estrada *et al.* 2004; Johansson *et al.* 2005). Manure applications have been reported to increase the abundance of clinically relevant antibiotic resistance genes and antibiotic-resistant bacteria in soil (Heuer, Schmitt and Smalla 2011; Zhou *et al.* 2013; Udikovic-Kolic *et al.* 2014; Mckinney *et al.* 2018; Wolters *et al.* 2018). It is worth mentioning that anthropogenic activities have released antibiotics and heavy metals into the aquatic and soil environment, which in small concentration exert selective pressure to both exogenous and native soil microbiota (Bürgmann *et al.* 2018; Xie, Shen and Zhao 2018). In this study, the copper tolerance test revealed a high MIC value (8 mM) of *A. baumannii* strains compared with others (Figure S10, Supporting Information). Genome analysis showed presence of copper-related efflux genes (*copA* and *copB*) among all *A. baumannii* strains of this study (Fig. 6; Figure S5, Supporting Information). The expression of these genes among *A. baumannii* strains could be the answer behind slightly higher copper tolerance compared with other *Acinetobacter* isolates. Williams *et al.* (2016) performed *in vitro* analysis in the presence of copper and showed the expression of copper-related genes (*copA* and *copB*) in clinical *A. baumannii* strains as well. Recent analysis of large complete genome collections revealed frequent co-occurrence of antibiotic and metal ion resistance genes among human pathogens compared with bacteria less often colonizing humans (Li, Xia and Zhang 2017). Members of *Acinetobacter* with a plasmid encoding a copper resistance gene (*copA*) have recently been reported (Irawati, Yuwono and Rusli 2016). Resistance to metal ions is often associated with mobile genetic elements that have been responsible for a wide dissemination of antibiotic resistance genes (Baker-Austin *et al.* 2006; Hobman and Crossman 2015). Furthermore, self-transferable plasmids conferring resistance toward multiple antibiotics were isolated from animal manure applied as fertilizer (Smalla *et al.* 2000; Heuer *et al.* 2002; Heuer and Smalla 2007). Indeed, as long as antibiotic resistance genes exist in soil and manure, the probability for their transfer remains obvious, and since a plethora of antibiotic resistance genes are exchanged in a single horizontal gene transfer event, this facilitates eventual evolution of multidrug-resistant strains from susceptible bacteria under appropriate selection pressure (Thomas and Nielsen 2005; Chee-Sanford *et al.* 2009; Hughes and Andersson 2015). It is now generally accepted that manured soil and plant phytosphere represent hotspots for horizontal gene transfer, most importantly between organisms associated together, presumably because of rich nutrients of manure (Van Elsas, Turner and Bailey 2003; Heuer and Smalla 2007; Chee-Sanford *et al.* 2009; Heuer, Schmitt and Smalla 2011; Nesme and Simonet 2015). Recently, Leclercq *et al.* (2016) reported environmental species of *Acinetobacter* among the group of genera involved in persistence of antibiotic resistance genes in manure treated soil. The application of effluent manure harboring potential pathogens obtained from anaerobic digestion processes in the agricultural fields might result in colonization of plants. Berg *et al.* (2002) and Sachdev *et al.* (2010) successfully isolated *A. baumannii* (strain LRVP52; GenBank: EU221389, strain LRFN53; GenBank: EU221350, and strain HIRFP40; GenBank: EU921471) from the rhizosphere of agricultural plants. The horizontal gene transfer of antibiotic resistance genes might occur when relevant donor bacteria are present in such niches, thus the movement of antibiotic resistance genes from pathogens into soil likely results in transfer and persistence of antibiotic resistance genes in natural environments once stably acquired in soil microbiota. This may lead to human infections with emerging pathogens, akin to the rise of *A. baumannii* infections (Chee-Sanford *et al.* 2009; Forsberg *et al.* 2012).

Members of the genus *Acinetobacter* mostly have optimum growth temperatures of 33–35°C but especially *A. baumannii* shows good growth at 42–45°C (Doughari *et al.* 2011). All six strains of *A. baumannii* were observed to survive at 50°C (Figure S9, Supporting Information). In a recent study, similar high growth temperatures have been observed by Hrenovic *et al.* (2014); a multidrug-resistant environmental *A. baumannii* strain reportedly grew at a high temperature (50°C), and showed 87% similarity (pulsed-field gel electrophoresis based molecular typing) to a clinical isolate of *A. baumannii* obtained from the general hospital in Pula (Croatia). These isolates represented a cluster within European clone I (IC 1) that had been recognized as a cause of nosocomial infection in Croatia for over 10 years. In addition, Yavankar, Pardesi and Chopade (2007) investigated 39 of 118 *Acinetobacter* spp. isolates from human epidermis and reported the tolerance of isolates at temperature of 50°C. As suggested by Hrenovic *et al.* (2014), the extracellular polymeric substances produced by *A. baumannii* may have an additional protective function under unfavorable environmental conditions (low pH, desiccation and high temperatures). Furthermore, the survival of all six *A. baumannii* strains and one isolate (namely KPC-SM-21) closely assigned to *A. baumannii* in high temperature zones (>40°C, mesophilic; originated from output samples) in German biogas plants of this study might be explained by biosynthesis of trehalose as a compatible solute by prokaryotes under environmental stress (McIntyre *et al.* 2007; Reina-Bueno *et al.* 2012). Recently, Zeidler *et al.* (2017) investigated temperature and salt induced solute implication in pathobiology of *A. baumannii* and reported that high osmolarity and high temperatures are important factors for expression of trehalose-6-phosphate phosphatase (*otsB*) and biosynthesis of trehalose in osmo- and thermotolerant wild type *A. baumannii* strains.

Currently, there exists only one study regarding the role of *Acinetobacter* in persistence of antibiotic resistance genes in manure amended soil (Leclercq *et al.* 2016). We assumed that both opportunistic and environmental strains of *Acinetobacter* may play a role in dissemination of antibiotic resistance genes and a detailed study is required to cover the total abundance of *Acinetobacter* spp. that can be released with manure and biogas plants digestate into the environment to understand the role played by *Acinetobacter* in spread of antibiotic resistance genes.

This study provided a first detailed characterization of *Acinetobacter* spp. released via manure and from biogas plants after anaerobic digestion, transferred through the anaerobic post-fermenter and storage tank into the environment. Because studied biogas plants were CSTR systems with a continuous inflow of fresh slurry and manure material, the given retention time gave just estimates how long the cultured output bacteria were exposed to anaerobic conditions. However, because the output samples were taken from the final storage tank containing the digestates used for field application without further treatments, those samples were the best option to determine the risk of pathogen release from biogas plants. Our study showed that *Acinetobacter* isolates closely associated to important nosocomial pathogens were present in manure and biogas plant digestates, both fertilizers used in agriculture. The strains of *A. baumannii* from this study had higher MIC values than other isolates for all tested antibiotics except cefquinome (with and without clavulanic acid), cefotaxime, ceftazidime (with or without avibactam or 3-aminophenylboronic acid), ceftolozan, amoxicillin, amikacin,

quinolones (enrofloxacin, levofloxacin and ciprofloxacin), tetracycline, trimethoprim/sulfamethoxazole, tigecycline, carbapenems (meropenem and imipenem) and colistin, respectively (Tables S3 and S4, Supporting Information). Although all *A. baumannii* strains lacked potent acquired antibiotic resistance genes, the comparative genome analyses showed the presence of multiple efflux-related and virulence or pathogenicity factor-related genes in their genomes, which are necessary for survival of this bacterium in both abiotic and biotic environments. Indeed, the cultivation-dependent method detected the survival of strains of *A. baumannii* in thermophilic and mesophilic anaerobic biogas digestion processes, which consequently pose significant environmental and epidemiological risks when released in agricultural soil via application of effluent manure. We support the use of both culture-dependent and culture-independent approaches to understand the diversity and role of *Acinetobacter* in anaerobic digestion plant and soil amended with the resulting digestate in a prospective study. Overall, this study has shown that isolates related to nosocomial *A. baumannii* were released from manure and digestates of anaerobic German biogas plants into the environment and agricultural land. More detailed cultivation studies with *Acinetobacter* selective media with and without antibiotics are required to get a better knowledge on the release of (antibiotic)-resistant *Acinetobacter* from livestock with and without anaerobic manure treatment. It is also not yet clear whether a thermophilic biogas plant process would have a better elimination efficiency than a mesophilic biogas plant process. This is however indicated because all cultured *Acinetobacter* spp. could not grow at 55°C and above.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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CHAPTER IV

Genomic plasticity and adaptive capacity of the quaternary alkyl-ammonium compound and copper tolerant *Acinetobacter bohemicus* strain QAC-21b isolated from pig manure

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ORIGINAL PAPER

Genomic plasticity and adaptive capacity of the quaternary alkyl-ammonium compound and copper tolerant *Acinetobacter bohemicus* strain QAC-21b isolated from pig manure

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Abstract Here, we present the genomic characterization of an *Acinetobacter bohemicus* strain QAC-21b which was isolated in the presence of a quaternary alky-ammonium compound (QAAC) from manure of a conventional German pig farm. The genetic determinants for QAAC, heavy metal and antibiotic resistances are reported based of the whole genome shotgun sequence and physiological growth tests. *A. bohemicus* QAC-21b grew in a species typical manner well at environmental temperatures but not at 37 °C. The strain showed tolerance to QAACs and copper but was susceptible to antibiotics relevant for *Acinetobacter* treatments. The genome of QAC-21b contained several *Acinetobacter* typical QAAC and heavy metal transporting efflux pumps coding genes, but no key genes for acquired antimicrobial resistances. The high genomic content of transferable genetic elements indicates that this bacterium can be involved in the transmission of antimicrobial resistances, if it is released with manure as organic fertilizer on agricultural fields. The genetic content of the strain was compared to that of two other *A. bohemicus* strains, the type strain ANC 3994^{T}, isolated from forest soil, and KCTC 42081, originally described as *A. pakistanensis,* a metal resistant strain isolated from a wastewater treatment pond. In contrast to the forest soil strain, both strains from anthropogenically impacted sources showed genetic features indicating their evolutionary adaptation to the anthropogenically impacted environments. Strain QAC-21b will be used as model strain to study the transmission of antimicrobial resistance to environmentally adapted *Acinetobacter* in agricultural environments receiving high content of pollutants with organic fertilizers from livestock husbandry.

Keywords *Acinetobacter bohemicus* · QAAC tolerance · Manure · Environmental transmission

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Introduction

Members of the genus *Acinetobacter* are widespread in nature and have been cultured from both clinical and non-clinical environments including soil, water, wild birds and domestic/farm animals (Towner 2009; Visca et al. 2011; Wilharm et al. 2017; Pulami et al. 2021). The ecology of most of the species is still not

well understood (Cool et al. 2019). Multi-drug resistant (MDR) pathogens belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex have immense clinical significance for causing hospital-associated nosocomial outbreaks globally (Wong et al. 2017; Vázquez-López et al. 2020). The overuse of antibiotics in the clinical environment and livestock husbandry has accelerated the dissemination of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from human populations and livestock into the receiving environment (Chee-Sanford et al. 2009; Rahube et al. 2012; Rizzo et al. 2013; Bürgmann et al. 2018; Xie and Zhao 2018). Beside antibiotic residues, heavy metals and biocides, including quaternary alkyl-ammonium compounds (QAACs), are present in wastewater and manure from livestock husbandry in parallel (Heuer et al. 2011; Rahube et al. 2012; Mulder et al. 2018; Kinney et al. 2006; Christou et al. 2017; Pan and Chu 2018). Those pollutants can trigger the spread of ARGs via co-selection processes in manure and WWTPs or in the environment where bacteria and pollutants come in close contact (Chapman 2003; Imran et al. 2019). Resistance and tolerance to QAACs and heavy metal ions is often associated with mobile genetic elements (MGEs) that have been responsible for a wide dissemination of ARGs (Baker-Austin et al. 2006; Hobman and Crossman 2015; Li et al. 2017). *Acinetobacter* spp. including *A. baumannii* strains have occasionally been isolated from raw manure and digested manure of biogas plant digestates (Schauss et al. 2015, 2016; Pulami et al. 2020). So far, less is known about QAAC resistance of *Acinetobacter* which are released with manure in the agricultural environment. Less is known about *Acinetobacter* from those sources which do not grow at pathogen relevant temperatures because cultivation studies for risk assessment are often performed at higher temperatures as 37 °C or even 44 °C (Schauss et al. 2015, 2016; Klotz et al. 2019; Pulami et al. 2020). We attempted to study the presence of QAAC tolerant bacteria which grew at environmental relevant temperatures in pig manure (data unpublished). The studied manure slurry contained QAACs and was used as fertilizer for agricultural fields. During this study we cultured an *Acinetobacter* strain, QAC-21b, in the presence of 50 µg benzyldimethyldodecylammonium chloride (BAC-C12) mL^{-1}. The strain was identified as a member of the species *Acinetobacter bohemicus.* The species was originally proposed based on 25 isolates which were isolated from different soil and water samples collected in natural ecosystems in the Czech Republic (Krizova et al. 2014). In the same year, a second species, *A. pakistanensis* was proposed based on one strain which was isolated from a textile dying wastewater treatment pond in Pakistan (Abbas et al. 2014). This strain was characterized as heavy metal resistant and psychrotolerant. *A. pakistanensis* was later reclassified as heterotypic synonym of *A. bohemicus* (Nemec and Radolfova-Krizova 2016). Based on phylogenomic analysis, *A. bohemicus* belongs together with the next related species "*A. kyonggiensis*", *A. albensis, A. harbinensis, A. terrestris*, *A. terrae* and *A. kookii* to a clade of *Acinetobacter* species which contains species that have nearly exclusively recovered from soil and water ecosystems (Clade G; Nemec 2022). As reported for most of the species of this clade, *A. bohemicus* strains did not grow at 37 °C (Nemec 2022). Strains of *A. bohemicus* just grew at 30 °C, but not at 35 °C in brain heart infusion broth (Krizova et al. 2014; Nemec and Radolfova-Krizova 2016).

Here we present a detailed physiological and genomic characterization of the BAC-C12 tolerant *A. bohemicus* strain QAC-21b isolated from the manure sample of a German pig farm. Genetic features explaining QAAC and heavy metal tolerance were studied. The presence of further antimicrobial resistance genes, insertion sequence (IS) elements, phages and pathogenicity genes were examined. Physiological tests were performed in parallel to confirm the genetic predictions of antimicrobial resistances. Aim of the detailed characterization was to understand the adaptation of the strain to the anthropogenic environment and to determine the potential risk associated with QAAC tolerant *Acinetobacter* if it is released on an agricultural field. Genome based analyses were performed in comparison to *A. bohemicus* ANC 3994^T, which was originally isolated from an environmental habitat which had no contact to anthropogenic pollutants and the copper tolerant strain KCTC 42081 (originally described as the type strain of *A. pakistanensis*) also isolated from an anthropogenically impacted environment.

Material and methods

Sampling, isolation and initial phylogenetic identification

The studied strain was isolated from a pooled manure sample collected in March 2017 from a manure storage tank on a pig farm in Hesse, Germany. The pigsty on the farm was cleaned with a mixture of water and didecyldimethylammonium chloride, and the mixture drained in the same tank as the manure. Three samples (each 40 mL) were taken in 50 mL sterile screw cup tubes (Greiner Bio-One GmbH, Germany) and transported cooled to 6 °C to the laboratory. Cultivation was performed during the same day. Bacteria were detached from 10 g liquid manure (pooled from the three replicates) by shaking the sample for 5 min at 25 °C in 90 mL 0.2% tetrasodiumpyrophosphate buffer (TSPP; 0.22 µm; filter-sterilized) in a sterile 250 mL glass bottle at 150 rpm on a horizontal shaker. Thereafter, the bottle was stored for 30 min in the dark to enable the sedimentation of manure particles. After sedimentation, cultivation was performed with the bacterial suspension (upper supernatant 30 mL: 10^{-1} dilution). A subsample of 0.5 mL was serially diluted (up to 10^{-3}) in 0.9% (w/v) NaCl solution. From each dilution 100 µL were plated on different agar media which were incubated for 72 h in the dark at 25 °C. A round, beige colony with a diameter of 1.5 mm was picked from Mueller–Hinton agar (MH, Carl-Roth, Germany) supplemented with 50 µg mL^{-1} BAC-C12. The colony was purified by streaking single colonies for multiple times and assigned as strain QAC-21b. Fresh biomass of the strain was preserved for long term storage in two 1.4 mL U-bottom push cap tubes (Micronic, Netherlands) with 500 µL Gibco new-born calf serum (NBCS, ThermoFisher Scientific) at −20 and −80 °C. The strain was assigned by partial 16S rRNA gene sequencing as described by Schauss et al. (2015) to the genus *Acinetobacter*. The Sanger sequenced 16S rRNA gene of strain QAC-21b was deposited in GenBank (NCBI) with accession number OM327586.

Physiological tests for taxonomic characterization

Phenotypic characterization of strain QAC-21b was performed with the API 20 NE system (bioMérieux). The cytochrome-c oxidase activity was tested with the Bactident oxidase test strips (Merck). Hemolysis activity was tested on Columbia agar with 5% sheep blood (SBA; Oxoid) as described by Krizova et al. (2014). All incubations were performed at 25 °C. Temperature dependent growth was tested with the spot assay technique as described by Pulami et al. (2021). Growth in brain heart infusion (BHI) broth (Sigma Aldrich) at 30 °C and 35 °C were performed according to Krizova et al. (2014) and Nemec and Radolfova-Krizova (2016).

Susceptibility tests against QAACs, copper and antibiotics

The two QAACs, BAC-C12 and didecyldimethylammonium chloride (DADMAC-C10), were used for susceptibility testing of the strain against common biocides present in disinfection solutions used on farms. Minimal inhibitory concentrations (MIC) values were determined by broth microdilution assay following the CLSI guidelines (M100-ED30) as described by Heyde et al. (2020). Following concentration ranges were tested, 0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg BAC-C12 mL^{-1}, and 0, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 µg DADMAC-C10 mL^{-1}. Strain QAC-21b was cultured on MH agar over night at 25 °C and suspended in 0.9% sterile NaCl to a turbidity equal to a 0.5 McFarland standard. 61 µL of the suspension was used for the inoculation of 14 mL MH broth. The broth microdilution assay was performed in a 96 wells plate (Greiner Bio-one) in a total volume of 150 µL per test well containing 25 µL double concentrated MH broth, 25 µL water dissolved QAACs (six-fold concentration) and 100 µL of the bacterial suspension in MH broth. The plate was sealed with sterile transparent plastic cover and incubated for 48 h at 25 °C under humid conditions. The lowest concentration that inhibited growth was considered as MIC value of the QAAC. Copper resistance was tested according to Pulami et al. (2020). Susceptibility test against antibiotics was performed in the MRGN Micronaut-S System panel (Merlin, Germany) and a Micronaut S panel (Merlin) containing mainly veterinary relevant antibiotics (Schauss et al. 2015, 2016) following the CLSI guidelines as described previously (Pulami et al. 2020). Classification into sensitive (S) or resistant (R) for antibiotics was done according to EUCAST (http://www.eucast.org/clinical breakpoints/) and CLSI (M100-ED30).

Genome sequencing, phylogenetic assignment, and genome-wide analyses

Genomic DNA was extracted with the MasterPure DNA purification kit (Epicentre, Madison, Wisconsin, USA) from fresh biomass cultured on MH agar at 25 °C. A DNA library was generated using the Nextera XT DNA sample preparation kit following the manufacturer's instructions. The whole genome shotgun library was sequenced using the dual index paired-end (v3, 2 × 300 bp) approach for the Illumina MiSeq platform as recommended by the manufacturer (Illumina, San Diego, USA). The genome assembly was performed as described previously (Pulami et al. 2021). The genome sequence was deposited in NCBI under accession number CAJJDZ000000000.

Initial phylogenetic analysis based on the partial 16S rRNA gene sequence was performed according to Pulami et al. (2021). A 16S rRNA gene at contig NZ_CAJJDZ010000011 (locus tag: QAC21B_03874) was identical to the Sanger sequence of the 16S rRNA gene and confirmed the strain assignment. For a higher resolution phylogenetic analysis, genes of the DNA-directed RNA polymerase β-subunit (*rpoB*) and the DNA gyrase β-subunit (*gyrB*) were retrieved from genome sequences. Analysis is described in detail in the Supplementary Material.

Comparative genomics including average nucleotide identity (ANI) analyses were performed in EDGAR 3.0 (Blom et al. 2016; Dieckmann et al. 2021). For comparative genomics, genome sequences of the type strains of *A. bohemicus*, *A. pakistanensis* (later heterotypic synonym of *A. bohemicus*; Nemec and Radolfova-Krizova 2016), *A. johnsonii* and *A. kookii*, and *A. baumannii* as well as some well characterized *A. baumannii* strains, AYE, ATCC 17987, and KPC-SM-125 were used. The latter one was isolated from digested manure of German biogas plant (Pulami et al. 2020).

Metal tolerance genes (MTGs) and antibiotic resistance genes were searched using BacMet database (Pal et al. 2014) and Resfinder 4.0 (Zankari et al. 2012; Bortolaia et al. 2020). IslandViewer 4 (Bertelli et al. 2017) for the GI-like regions, and ISfinder (Siguier et al. 2006) was applied to search IS elements. Phage-related sequences were searched using PHASTER (Arndt et al. 2016; Zhou et al. 2011) and plasmids via Plasmidfinder 2.1 (Carattoli et al. 2014). Virulence factor database (VFDB) was used to identify virulence genes (Chen et al. 2005; Liu et al. 2022). Easyfig v2.2.5 (Sullivan et al. 2011) was used to visualize the comparisons of efflux pump operons detected in the genome.

Results and discussion

Phylogenetic assignment of strain QAC-21b to the species *A. bohemicus*

The initial phylogenetic identification of QAC-21b showed that the strain shared highest 16S rRNA gene sequence similarity with the type strain of *A. bohemicus* (99.72%) and was placed into the distinct cluster of all *A. bohemicus* strains in a phylogenetic trees based on the 16S rRNA gene (Figure S1, Supplementary data). This was confirmed by the phylogenetic analyses based on the common *Acinetobacter* marker genes *rpoB* and *gyrB* (Figures S2–S4, Supplementary data).

Phylogenomic analysis finally confirmed the species assignment of QAC-21b. ANI values between the genome sequence of QAC-21b and those of *A. bohemicus* ANC 3994^T and KCTC 42081 were 98.0 and 98.9%, respectively (Figure S5a). The values were above the 95% threshold for species discrimination (Richter and Rosselló-Móra 2009), and within the range of intraspecies ANI values described for *A. bohemicus* (95.92–96.08%) (Nemec and Radolfova-Krizova 2016). The ANI values indicated that QAC-21b represents a novel strain of the species *A. bohemicus*.

Comparative genome data

The genome size (3.99 Mbp) and the GC content (39.4%) of the whole genome sequence (WGS) of strain QAC-21b were in a similar range to that of ANC 3994^T (3.66 Mbp and 39.6%) and KCTC 42081 (3.7 Mbp and 39.2%) (Table 1). The genome of QAC-21b contained 3943 predicted genes and 3848 coding DNA sequence (CDS). Additional genomic features are provided in Table 1. Strain QAC-21b shared 2639 of 3535 genes (74.5% of the genomic gene content) with the other two strains of

Table 1 Genome assembly and annotation statistics of *A. bohemicus* strains QAC-21b, ANC 3994^T and KCTC 42081

Genome features	QAC-21b	ANC 3994^T	KCTC 42081
GenBank accession number	NZ_CAJJDZ000000000	NZ_APOH00000000	NZ_FOZU00000000
Approximate genome size	3,996,158 bp	3,647,901 bp	3,728,266 bp
BioProject number	PRJEB42457	PRJNA224116	PRJNA224116
BioSample number	SAMEA7810071	SAMN01828192	SAMN05444586
GC content	39.4%	39.6%	39.2%
Genome coverage	22×	87.0×	413×
Number of contigs	17	28	105
Contig N50	902,613 bp	881,473 bp	85,922 bp
Contig L50	2	2	12
Number of scaffolds	17	14	104
Longest contig (bp)	1,907,865 bp	794,304 bp	301,207 bp
Number of predicted genes	3943	3430	3638
Number of predicted CDS	3848	3320	3559
Number of complete rRNAs	11	24	3
Number of partial rRNAs	1	Absent	Absent
Number of predicted tRNAs	78	82	72
Number of non-coding RNAs	5	4	4
Number of pseudogenes	203	72	188
Source	Animal (pigs)	Natural environment	Textile dying wastewater treatment pond
Sample	Pig manure	Deciduous forest soil	Wastewater pond
Country	Germany	Czech Republic	Pakistan
Publication	This study	Krizova et al. (2014)	Abbas et al. (2014)

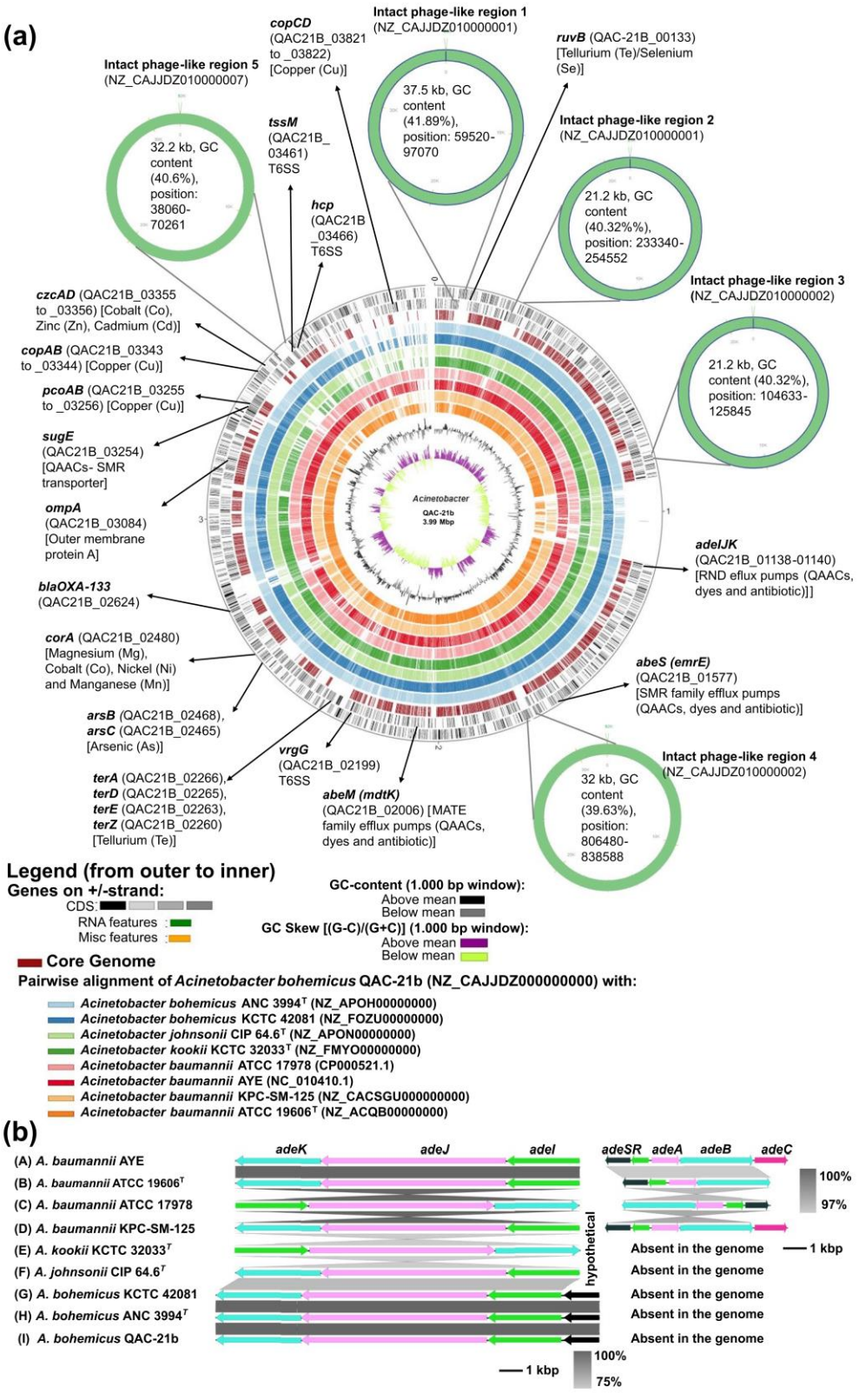
the species *A. bohemicus*. Strain QAC-21b shared 77 gene (2.2%) only with ANC 3994^T and a higher proportion of gene, 307 genes (8.7%), only with KCTC 42081 (Figure S5b). A total of 519 genes (14.7%) were only detected in the genome of strain QAC-21b. The percentage of genes specific for individual strains was similarity for the two other studied strains.

Physiological properties of strain QAC-21b confirming species assignment

Physiological properties of strain QAC-21b confirmed the species assignment. Strain QAC-21b was cytochrome-c oxidase negative and did not produce acid from D-glucose, was negative for gelatine hydrolysis and nitrate reduction (API 20 NE system) (Krizova et al. 2014; Nemec and Radolfova-Krizova 2016). Strain QAC-21b was positive for esculin hydrolysis (API 20 NE system) and did not assimilate D-glucose, L-arabinose, malate, phenylacetic acid, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid and trisodium citrate. A slight hemolysis was obtained on sheep blood agar. Good growth was observed at 30 °C but not at 35 °C in BHI broth. Spot assays showed an optimum temperature range for growth of QAC-21b between 25 to 30 °C, growth was strongly reduced at 15 °C and no growth was observed at 37 °C (Figure S6). The results were confirm to those reported by Krizova et al. (2014) for *A. bohemicus* strains, most of which showed a weak hemolysis of sheep blood and did not grow at 35 °C.

Phenotypic resistance of strain QAC-21b to QAAC, copper and common antibiotics

MIC values of QAC-21b for BAC-C12 and DADMAC-C10 were 50 and 2.5 µg mL^{-1}. The strain grew on MH agar supplemented with 4 mM copper, but not


(a)
Intact phage-like region 1 (NZ_CAJJDZ010000001)
copCD (QAC21B_03821 to _03822) [Copper (Cu)]
ruvB (QAC-21B_00133) [Tellurium (Te)/Selenium (Se)]
Intact phage-like region 5 (NZ_CAJJDZ010000007)
37.5 kb, GC content (41.89%), position: 59520-97070
Intact phage-like region 2 (NZ_CAJJDZ010000001)
tssM (QAC21B_03461) T6SS
32.2 kb, GC content (40.6%), position: 38060-70261
hcp (QAC21B_03466) T6SS
21.2 kb, GC content (40.32%%), position: 233340-254552
Intact phage-like region 3 (NZ_CAJJDZ010000002)
czcAD (QAC21B_03355 to _03356) [Cobalt (Co), Zinc (Zn), Cadmium (Cd)]
copAB (QAC21B_03343 to _03344) [Copper (Cu)]
21.2 kb, GC content (40.32%), position: 104633-125845
pcoAB (QAC21B_03255 to _03256) [Copper (Cu)]
sugE (QAC21B_03254) [QAACs- SMR transporter]
Acinetobacter QAC-21b 3.99 Mbp
ompA (QAC21B_03084) [Outer membrane protein A]
adeIJK (QAC21B_01138-01140) [RND eflux pumps (QAACs, dyes and antibiotic)]]
blaOXA-133 (QAC21B_02624)
corA (QAC21B_02480) [Magnesium (Mg), Cobalt (Co), Nickel (Ni) and Manganese (Mn)]
abeS (emrE) (QAC21B_01577) [SMR family efflux pumps (QAACs, dyes and antibiotic)]
arsB (QAC21B_02468), arsC (QAC21B_02465) [Arsenic (As)]
vrgG (QAC21B_02199) T6SS
Intact phage-like region 4 (NZ_CAJJDZ010000002)
terA (QAC21B_02266), terD (QAC21B_02265), terE (QAC21B_02263), terZ (QAC21B_02260) [Tellurium (Te)]
abeM (mdtK) (QAC21B_02006) [MATE family efflux pumps (QAACs, dyes and antibiotic)]
32 kb, GC content (39.63%), position: 806480-838588
Legend (from outer to inner)
Genes on +/-strand:
CDS:
RNA features
Misc features
GC-content (1.000 bp window):
Above mean
Below mean
GC Skew [(G-C)/(G+C)] (1.000 bp window):
Above mean
Below mean
Core Genome
Pairwise alignment of Acinetobacter bohemicus QAC-21b (NZ_CAJJDZ000000000) with:
Acinetobacter bohemicus ANC 3994T (NZ_APOH00000000)
Acinetobacter bohemicus KCTC 42081 (NZ_FOZU00000000)
Acinetobacter johnsonii CIP 64.6T (NZ_APON00000000)
Acinetobacter kookii KCTC 32033T (NZ_FMYO00000000)
Acinetobacter baumannii ATCC 17978 (CP000521.1)
Acinetobacter baumannii AYE (NC_010410.1)
Acinetobacter baumannii KPC-SM-125 (NZ_CACSGU000000000)
Acinetobacter baumannii ATCC 19606T (NZ_ACQB00000000)
(b)
adeK
adeJ
adeI
adeSR
adeA
adeB
adeC
(A) A. baumannii AYE
(B) A. baumannii ATCC 19606T
(C) A. baumannii ATCC 17978
(D) A. baumannii KPC-SM-125
(E) A. kookii KCTC 32033T
(F) A. johnsonii CIP 64.6T
(G) A. bohemicus KCTC 42081
(H) A. bohemicus ANC 3994T
(I) A. bohemicus QAC-21b
hypothetical
100%
97%
Absent in the genome
1 kbp
100%
75%

◂**Fig. 1** **a** Circular plot of the whole genomes of *A. bohemicus* QAC-21b (size at the centre of plot), *A. bohemicus* ANC 3994^T, *A. bohemicus* KCTC 42081 (formerly type strain of *A. pakistanensis*), *A. kookii* 32033^T, *A. johnsonii* CIP 64.6^T, *A. baumannii* KPC-SM-125, *A. baumannii* ATCC 17,978, *A. baumannii* AYE, and *A. baumannii* ATCC 19606^T generated with BioCircos (Cui et al. 2016) implemented in EDGAR3 (Dieckmann et al. 2021). All labelled genes were found to be located at the chromosomes. **b** Comparision of operon of RND efflux pumps (AdeABC/AdeRS and AdeIJK) present in the chromosome of *Acinetobacter* strains, including *A. bohemicus* QAC-21b. Figure and comparison were visualized using Easyfig v2.2.5 (Sullivan et al. 2011)

in the presence of 8 mM copper and above (Figure S7). Strain QAC-21b showed intermediate resistance to tetracycline. However, it was susceptible against amikacin, cefotaxime, ceftazidime, ciprofloxacin, levofloxacin, colistin, imipenem, meropenem, piperacillin, piperacillin/tazobactam and trimethoprim/sulfamethoxazole (Table S1). It showed high MIC values for fosfomycin, oxacillin, ceftiofur (third generation chepalosporine), tylosin and florfenicol (Table S1).

QAACs and heavy metal efflux, and antimicrobial resistance genes

The genetic determinant often mentioned in context of QAAC tolerance is the gene *qacE* or its mutated version *qacEΔ1* (Gaze et al. 2005, 2011). These genes are often co-located with a class 1 integron on plasmids and correlated with reduced susceptibility to QAACs (Fournier et al. 2006; Gomaa et al. 2017; Lin et al. 2017). Both genes were found in clinically relevant *Acinetobacter*, mostly *A. baumannii*. Comparative genomics showed that neither strain QAC-21b nor the next related tested *Acinetobacter* strains contained those genes; *qacEΔ1*, co-located with a class 1 integron, was only detected in the *A. baumannii* MDR strain AYE which was isolated from an infected patient (Poirel et al. 2003) (Table S2, Figure S8). The genetic arrangement found in that strain was typical for that gene.

Search in the BacMet database and comparative genomics showed several genes encoding multiple QAACs and antimicrobial efflux pumps such as multidrug and toxic compound extrusion (MATE) family efflux pumps (*abeM* or *mdtK*), small multidrug resistance (SMR) family efflux pumps (*sugE* and *abeS*) and resistance-nodulation-cell division (RND) efflux pump (*adeIJK*) in the genome of strain QAC-21b and the other *Acinetobacter* strains used for comparative analyses (Fig. 1A, B; Table S2). The combined action of these efflux pumps might be responsible for reduced susceptibility to the tested QAACs. Previous studies showed the association of *adeIJK* with reduced susceptibility to benzalkonium chloride (QAAC) in clinical *A. baumannii* strains (Damier-Piolle et al. 2008; Rajamohan et al. 2010; Lin et al. 2017). *AbeM* is known to be associated with efflux of QAAC in *Acinetobacter* (Su et al. 2005; Lin et al. 2017). Similarly, *abeS* was known to be associated with reduced susceptibility to benzalkonium chloride in clinically associated *A. baumannii* strain AC0037 (Srinivasan et al. 2009). *SugE* was associated with reduced susceptibility to benzalkonium chloride in *Escherichia coli* (Chung et al. 2002; He et al. 2011). We could not determine genes of the RND type efflux pumps AdeABC and AdeRS in the genome of QAC-21b and next related strains, but in all studied *A. baumannii* strains including strain KPC-SM-125, which was isolated from digested manure.

Analysis in BacMet database revealed metal efflux pump genes such as tellurium/selenium efflux genes (*terZ, terE, terD, terA* and *ruvB*), magnesium/cobalt/nickel/manganese efflux genes (*corA*), arsenic efflux genes (*arsC* and *arsB*), zinc/cobalt/cadmium efflux genes (*czcA* and *czcD*), copper resistance protein efflux genes (*pcoA, pcoB, copA, copB, copC* and *copD*) in the genome of QAC-21b (Fig. 1A; Table S2). These genes were reported among the copper tolerant strains of *A. baumannii* (Williams et al. 2016; Thummeepak et al. 2020). Other heavy metals were not tested so far. Tellurium resistance genes were here only detected in strain QAC-21b and the two other *A. bohemicus* strains, but not in the genomes of the other studied strains (Table S2).

The resistome analysis showed that the genome of QAC-21b harbored one gene coding for an intrinsic class D beta-lactamase (Table S2). The high MIC value against oxacillin may be explained by the expression of intrinsic class D β-lactamase gene present in the genome of *Acinetobacter* species (Cho et al. 2018). Naturally weakly expressed class D β-lactamases ($bla_{\text{OXA-type}}$) become potent if insertion of an IS element occurs upstream (Vandecraen et al. 2017), however QAC-21b lacked an IS element upstream of this gene. The complete sequence of the intrinsic class D beta-lactamase (blaOXA-133, assigned via NCBI genome submission) of QAC-21b

had 96.43% (nucleotide based) and 95.90% (protein based) similarity to the intrinsic beta-lactamase of ANC 3994^T (*bla*OXA-296, locus tag: F994_00492). The intrinsic class D beta-lactamase of KCTC 42081 was disrupted by an internal stop codon and not assigned further. Perichon et al. (2014) identified a second intrinsic beta-lactamase gene ADC-ANC 3994 (AmpC-type) in the genome of ANC 3994^T (logus tag F994_00202). A homologue to this gene was not present in the genomes of QAC-21b and KCTC 42081.

No specific ARGs were determined in the genome of strain QAC-21b. The higher MIC values for the third-generation cephalosporin ceftiofur, florfenicol, fosfomycin and tylosin, and intermediate resistance to tetracycline (Table S1) could be due to the basal-level expression of *adeIJK* and *abeM* (reviewed by Coyne et al. 2011). The intrinsic resistance of *Acinetobacter* to these veterinary (florfenicol and ceftiofur) and clinical (fosfomycin) antibiotics is known (Coyne et al. 2011), however few studies had been performed to use fosfomycin in combination with other drugs, such as colistin, minocycline and polymyxin against *Acinetobacter* (Zhang et al. 2013; Sirijatuphat and Thamlikitkul 2014).

Plasmid and phage content

The draft genome sequence of QAC-21b lacked plasmids, but contained several phages integrated into the genome. Five intact, five incomplete, and three questionable phage-like regions were detected in the chromosome using PHASTER (Table S3). Similarly, ANC 3994^T showed one intact, five incomplete and two questionable phage-like regions (Table S4). Only four incomplete phage-like regions were found in the genome of KCTC 42081 (Table S5). The intact phage-like regions in QAC-21b were similar to the *Mannheimia* phage vB_MhM_3927AP2 (NC_028766.1) and the *Acinetobacter* phage YMC11/11/R3177 (NC_041866.1). In contrast, the intact phage-like region in ANC 3994^T was similar to *Burkholderia* phage KS14. The two intact phage-like regions located in contigs NZ_CAJJDZ010000001 (phage region size: 37.5 kb) and NZ_CAJJDZ010000002 (phage region size: 32 kb) of strain QAC-21b carried genes coding for integrases, transposases, terminases, tRNAs, and phage portal, head, tail, plate, fibre and phage-like proteins, including the *attL* and *attR* recognition sites, and multiple hypothetical genes (Figs. 1A, S10). By contrast, the phage region detected in the genome of ANC 3994^T lacked genes coding for integrase, transposase and terminase, and the *attL* and *attR* recognition sites were absent (Figure S10). Those genes are required for termination, integration, propagation and lysis inside the host bacterium (Casjens 2003; Canchaya et al. 2003; Labrie et al. 2010). The presence of several phage related genes integrated into the genome of strain QAC-21b was in agreement with the previous findings of multiple phage-linked DNA regions in the genome of *Acinetobacter* species (Touchon et al. 2014). The presence of multiple genes encoding hypothetical proteins in all phage-like regions of strain QAC-21b might be associated with environmental (pig manure) adaptation. These phage-like regions help bacteria to gain antimicrobial resistance, adaptation across changing environments, and can provide novel virulence characteristics to the host bacterium (Brüssow et al. 2004). Multiple studies had reported presence of phages in genome of members of the genus *Acinetobacter*, for instance, *A. baumannii* was considered as polylysogenic as several strains harbored multiple integrated phages in the genome (Snitkin et al. 2011; Touchon et al. 2014; Badawy et al. 2020; Loh et al. 2020).

IS elements and genomic islands (GIs)

Analysis in the ISfinder showed that strain QAC-21b possessed putative IS elements with 98%, 97% and 98% amino acid sequence similarities to IS*Aba12*, IS*Aba14* and IS*Alw3* (Table S1). These IS elements are often found in the host *A. baumannii* and *A. lwoffii* (Montaña et al. 2016; Liu et al. 2020). IS*Aba14* was reported in clinically relevant European clone II of *A. baumannii* (Šeputienė et al. 2012). Previous studies had shown that IS*Aba12* was responsible for mobilization of the active miniature inverted-repeat transposon element (MITE, designated MITE$_{Aba12}$) in the genome of *A. baumannii* ATCC 17978^T, and disruption of the histone-like nucleoid structuring (*hns*) gene by IS*Aba12* resulted in multiple phenotypic alterations, including hypermotility (Eijkelkamp et al. 2013; Adams and Brown 2019).

Based on at least one method of island detection, SIGI-HMM (Waack et al. 2006) or IslandPath-DIMOB (Hsiao et al. 2003), in IslandViewer4, 14

putative genomic islands (GIs) were detected in the genomes of QAC-21b, 23 in the genome of ANC 3994^T and 21 in the genome of KCTC 42081. GIs of QAC-21b and KCTC 42081 carried genes encoding metal efflux pumps (for e.g., *czcA*, *czcD*, czcO, *pcoA, cusR, cusS, copA, copB, arsH*), antimicrobial resistance (for e.g., *mdtB* and *mdtC*), transposases, integrase, virulence-associated and hypothetical proteins (Table S6) but those of ANC 3994^T mostly contained genes coding hypothetical proteins and only a single transposase gene (IS*605* OrfB family; locus tag: F994_02674, TableS6). Microbial GIs are clusters of genes involved in the genome evolution and environmental adaptation. GIs are linked with symbiosis, metabolism, fitness, antimicrobial resistance and pathogenicity (Juhas et al. 2009). The mobile genetic elements (for e.g., transposon, IS elements, plasmids and pathogenicity island) are associated with contemporary rise of antibiotic resistance in *Acinetobacter* species, particularly those representing ACB complex (Peleg et al. 2008; Lean and Yeo 2017). The overview of genes associated with metabolism, transposon, IS elements, virulence, metal efflux pumps and antibiotic resistance present within GIs is provided in Fig. 2 and Table S6. There are several evidences that the spontaneous evolution of ARGs within the genus *Acinetobacter* has been facilitated by IS elements (Turton et al. 2006), integrons (Hujer et al. 2006), conjugative elements (Goldstein et al. 1983) and transformation (Wright et al. 2014).

Pathogenicity related genes

Strain QAC-21b as well as the next related studied strains possessed a type six secretion system (T6SS). This is a general feature reported from members of genus *Acinetobacter* (Weber et al. 2013, 2016; Lewis et al. 2019). It was reported that *Acinetobacter* often uses T6SS to compete against other bacteria (Weber et al. 2013; Carruthers et al. 2013). The T6SS gene locus in the genome of QAC-21b contained genes encoding the hemolysin coregulated protein Hcp, the valine-glycine repeat protein G (VgrG), the membrane spanning complex (TssM), the baseplate components TssE, TssF and TssG and sheath components TssB and TssC. Apart from T6SS, the search in the virulence factor database showed the presence of other pathogenicity genes encoding outer membrane protein A (OmpA), phospholipase D (PlcD) and two component response regulator transcription factor (BmfRS) (Table S1). Proteins expressed by these genes are involved in adherence, invasion, apoptosis, biofilm, persistence, pathogenesis and serum resistance (Choi et al. 2005, 2008a, 2008b; Gaddy et al. 2009; Lee et al. 2010; Smani et al. 2014; Wang et al. 2014; Jacobs et al. 2010; Tomaras et al. 2008; Liou et al. 2014).

Survival of anaerobic conditions in the manure storage tank

Several studies showed that the obligate aerobic *Acinetobacter* survived the anaerobic treatment processes in wastewater treatment plants (Higgins et al. 2018) and biogas plants (Schauss et al. 2015, 2016; Pulami et al. 2020, 2021). The slurry tank in the pig farm of this study also represented an anaerobic environment. Comparative genomics showed the presence of genes encoding AMP phosphotransferase (locus tag: QAC21B_01400) and adenylate kinase (QAC21B_01250) in the genome of QAC-21b. Combined enzymatic actions of these proteins help using accumulated phosphates inside the cells as energy source (Van Groenestijn et al. 1987). This explains the survival of obligate aerobic *Acinetobacter* at oxygen limited environments (Pulami et al. 2020, 2021) as it was also expected in the manure slurry tank where the strain was isolates from.

Knowledge gap on QAAC resistance of *Acinetobacter*

QAACs are common ingredients of pesticides, disinfectants and detergents in agriculture and animal husbandry (Mulder et al. 2018). As reported by the German Society of Veterinary Medicine, at least one quarter of disinfectants used contained QAACs (DVG 2015). Bioavailable concentrations of QAACs can result in cross-resistance (Singer et al. 2016) and co-selection of resistance to antibiotics and QAACs (Webber et al. 2015). However, this important role of QAAC resistance for AMR spread is known less, and little is known about resistance levels developed against QAACs. There is still a gap of knowledge at which point a bacterium must be considered as QAAC susceptible or QAAC resistant to decide if studies on

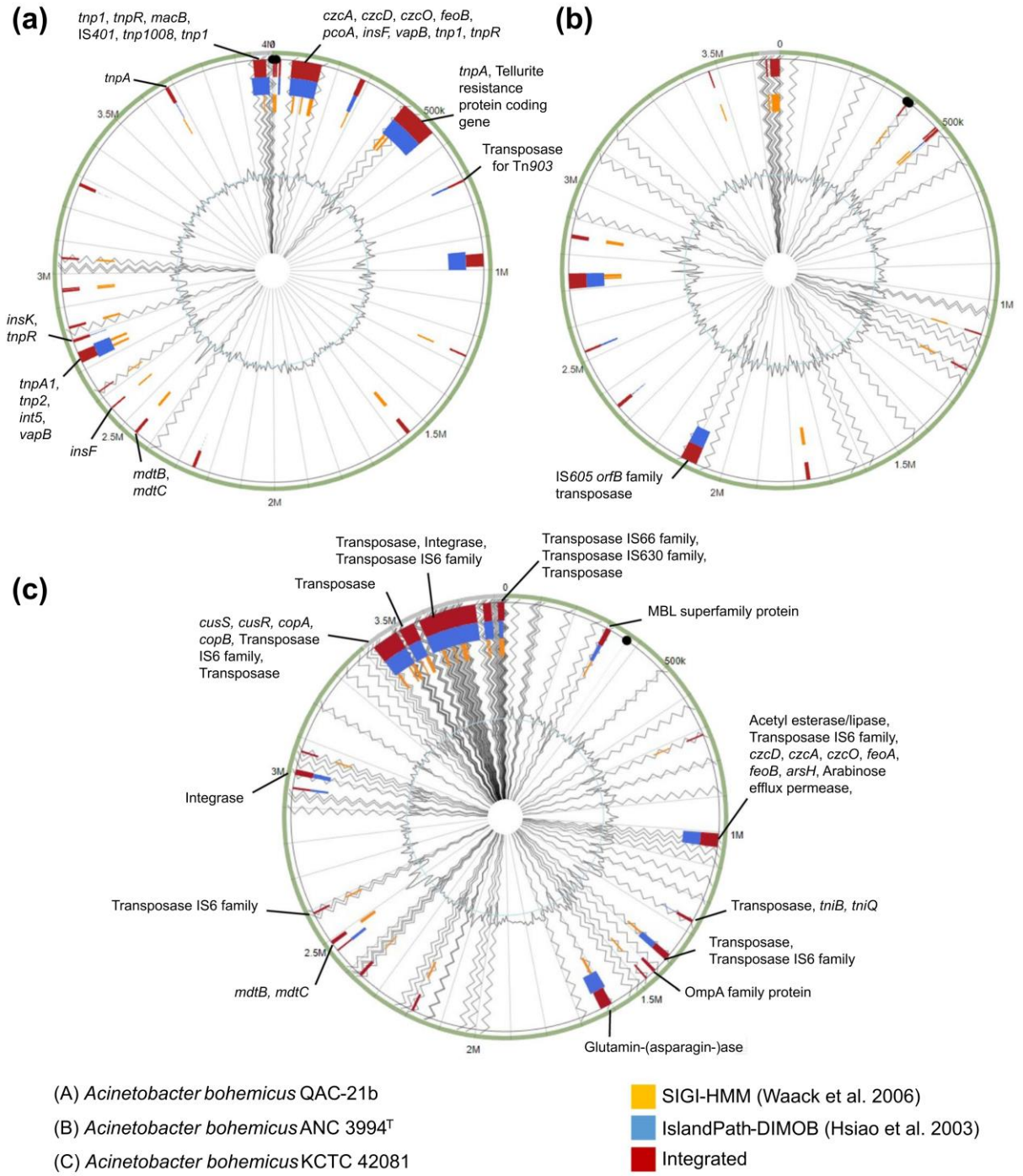


Fig. 2 Distribution of putative genomic islands (GIs) in *A. bohemicus* strains QAC-21b (**a**), ANC 3994^T (**b**) and KCTC 42081 (**c**). Only, genes associated to metabolism, transposon, IS elements, virulence, metal efflux pumps and antibiotic resistance present within GIs were shown. Genomic islands, which were not labelled, possessed hypothetical and other functional genes. The figures were generated with IslandViewer4. The complete genome of *A. baumannii* ATCC 19606^T (NC_CP045110) present in the database of IslandViewer4 was used as reference genome (represented by inner grey circles in the figure). Contig edges were shown by zigzag lines running from the center to the periphery

resistance mechanisms are relevant. No respective studied for *Acinetobacter* are published yet. We studied here only one strain and determined MICs for two common QAACs, BAC-C12 and DADMAC-C10. In a recent study we determined the effects of BAC-C12 and DADMAC-C10 exposure of an *A. baumannii* strain KPC-SM-21 isolated from digested manure (Heyde et al. 2020; Pulami et al. 2021). This strain had lower MIC values for the two QAACs (< 5 µg BAC-C12 ml^{-1} and 5 µg DADMAC-C10 ml^{-1}) than detected here for strain QAC-21b. This may indicate an increased tolerance of QAC-21b to both QAACs. Previous studies which reported on QAAC resistance mechanisms of *Acinetobacter* strains (Srinivasan et al. 2009; Rajamohan et al. 2010; Lin et al. 2017) did not consider pure QAAC compounds but worked with undefined mixtures of QAACs of different side carbon chain length, which made a direct comparison of our data with those of other studies not possible.

Conclusion

It has been already indicated that *Acinetobacter* species might contribute to spread and persistence of antimicrobial resistance in manured soil (Leclercq et al. 2016). More detailed studies are still required to understand those processes in detail. Here, we characterized a potential model strain isolated from manure which showed QAAC and copper-tolerance but was still antibiotic susceptible. However, the strain showed a high genetic potential to integrate receiving ARGs into its genome. The ability of QAC-21b to grow in a species typical manner well at environmental temperatures but not at 37 °C as typical human pathogenic *Acinetobacter* makes this strain a good model candidate for those environmental studies. Environmentally adapted strains are often neglected in risk assessments of antimicrobial resistance spread, but the presented genomic plasticity of QAC-21b shows that those strains should be considered as vector of transferable resistance determinants which is of concern in the current era of continuous rise in antibiotic resistances influenced by increased anthropogenic activities (Alanis 2005; Ji et al. 2012; Seiler and Berendonk 2012; Furlan et al. 2019). Differences in the structure of GIs and IS elements present in the *A. bohemicus* strains from anthropogenically impacted environments, compared to the type strain of *A. bohemicus* isolated from a native environment indicate adaptation of specific *Acinetobacter* strains of environmental species to human impacted environments. However further proofs including more strains are necessary. Our study indicated that more extensive studies of environmentally adapted *Acinetobacter* strains must be considered to understand the evolutionary adaptation of *Acinetobacter* to anthropogenic contaminations in the environment in much more detail. Strain QAC-21b can serve as a well characterized model strain for those studies.

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Authors' contributions D.P. performed the experiments and data analysis; S.G. supported data analysis; G.W. provided the genome sequence; J.B. and O.S. contributed to genome analyses; S.G. and D.P. wrote the manuscript; D.P. prepared all figures. All authors reviewed and contributed to writing to the manuscript.

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Availability of data and materials The Sanger sequenced 16S rRNA gene sequence and the whole genome shotgun sequence of QAC-21b are available at GenBank/EBML/DDBJ under Accession numbers OM327586 and CAJJDZ000000000.

Declarations

Competing interests The authors declare interests of a financial or personal nature.

Ethical approval Not applicable. No human and/ or animals were studies.

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CHAPTER V

Acinetobacter stercoris sp. nov. isolated from output source of a mesophilic german biogas plant with anaerobic operating conditions

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Contributions:

DP and SG designed the study and wrote manuscript which was proofed by all co-authors. TS was responsible for the isolation of the strain. DP and SG performed molecular analysis; DP physiological tests. TE provided MALDI-TOF data, PK fatty acid data, JB and GW genome sequence data data, respectively. OS, JB, DP performed genome annotation and/or genome sequence based analyses. Funding Open Access funding enabled and organized by Projekt DEAL. Sampling and cultivation of the studied strains was performed as part of the project RiskAGuA (02WRS1274A) funded by the Federal Ministry of Education and Research, Germany. Dipen Pulami was supported by Postgraduate Scholarships from Justus Liebig University Giessen.

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ORIGINAL PAPER

Acinetobacter stercoris sp. nov. isolated from output source of a mesophilic german biogas plant with anaerobic operating conditions

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Abstract The Gram-stain-negative, oxidase negative, catalase positive strain KPC-SM-21^T, isolated from a digestate of a storage tank of a mesophilic German biogas plant, was investigated by a polyphasic taxonomic approach. Phylogenetic identification based on the nearly full-length 16S rRNA gene revealed highest gene sequence similarity to *Acinetobacter baumannii* ATCC 19606^T (97.0%). Phylogenetic trees calculated based on partial *rpoB* and *gyrB* gene sequences showed a distinct clustering of strain KPC-SM-21^T with *Acinetobacter gerneri* DSM 14967^T = CIP 107464^T and not with *A. baumannii*, which was also supported in the five housekeeping genes multilocus sequence analysis based phylogeny. Average nucleotide identity values between whole genome sequences of strain KPC-SM-21^T and next related type strains supported the novel species status. The DNA G + C content of strain KPC-SM-21^T was 37.7 mol%. Whole-cell MALDI-TOF MS analysis supported the distinctness of the strain to type strains of next related *Acinetobacter* species. Predominant fatty acids were $C_{18:1}$ ω9c (44.2%), $C_{16:0}$ (21.7%) and a summed feature comprising $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH (15.3%). Based on the obtained genotypic, phenotypic and chemotaxonomic data we concluded that strain KPC-SM-21^T represents a novel species of the genus *Acinetobacter*, for which the name *Acinetobacter stercoris* sp. nov. is proposed. The type strain is KPC-SM-21^T (= DSM 102168^T = LMG 29413^T).

The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA and *rpoB*/*gyrB* gene sequences of strain KPC-SM-21^T are MT138756, MT157627 and MT157661, respectively. The GenBank/EMBL/DDBJ accession number of the whole genome shotgun sequence of strain KPC-SM-21^T is OOGT00000000; the version described in this paper is version OOGT01000000. Supplementary figures and tables are provided in online supplementary materials.

Supplementary Information The online version of this article (https://doi.org/10.1007/s10482-021-01517-7) contains supplementary material, which is available to authorized users.

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Keywords *Acinetobacter stercoris* · 16S rRNA · Genome · Biogas plant digestate

Abbreviations

MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
ANI	Average nucleotide identity
MLSA	Multilocus sequence analysis

Introduction

The genus *Acinetobacter* is highly diverse (Touchon et al. 2014) and was first described by Brisou and Prévot (1954). Members of this genus are Gram-negative coccobacilli, non-motile, non-spore forming, aerobic, oxidase negative and catalase positive bacteria. This genus comprises non-fermentative bacteria, which can survive under different environmental conditions for extended periods through a wide temperature range. Over the past decades, some species of this genus have emerged as significant nosocomial and opportunistic pathogens causing outbreaks of colonization and infection, especially in critically ill patients with impaired immunity (Dijkshoorn et al. 2007; Peleg et al. 2008; Towner 2006; Visca et al. 2011). Accumulation of antibiotic resistances in *Acinetobacter* spp. is an increasing problem for the global public health (Visca et al. 2011). *Acinetobacter baumannii* represents one of the "ESKAPE pathogens" which can cause life-threatening nosocomial infections and can harbor several drug resistance mechanisms (Rice 2008; Bush and Jacoby 2010). At the time of writing, the genus *Acinetobacter* comprised 59 distinct species with validly published names (https://lpsn.dsmz.de/genus/acinetobacter; Parte 2018), as well as several species and genomic species without validly published names. Most of the species of *Acinetobacter* were obtained exclusively from human clinical specimens (Nemec et al. 2001, 2003, 2010, 2011, 2015, 2016, 2017). However, others were isolated from environmental sources, such as activated sludge (Carr et al. 2003), wetlands (Anandham et al. 2010), forest soil (Kim et al. 2008), seawater (Di Cello et al. 1997; Vaneechoutte et al. 2009), dumpsites (Malhotra et al. 2012), wastewater (Vaz-Moreira et al. 2011), freshwater (Li et al. 2014; Radolfova-Krizova et al. 2016), cotton and soil (Nishimura et al. 1988; Choi et al. 2013). Furthermore, Rafei et al. (2015) reported as many as 30 putative novel species of *Acinetobacter* in a non-human epidemiological study in Lebanon, which suggested that this genus is geographically more distributed than originally supposed.

In an attempt to isolate carbapenem-resistant bacteria released from biogas plants (anaerobic processing condition) digestates into the environment, strain KPC-SM-21^T was isolated in October 2013 from the digestate collected from one of the studied German biogas plants (Schauss et al. 2015). Here, detailed phenotypic, genotypic and chemotaxonomic studies of strain KPC-SM-21^T were performed and the taxonomic status was concluded. Based on morphological, physiological, biochemical and genotypic characteristics obtained on the notion of a polyphasic approach, we propose a novel species of the genus *Acinetobacter* with strain KPC-SM-21^T as type strain. Besides, genes encoding antibiotic resistance, virulence and bacteriophages were identified, and survival of this strain in anaerobic condition was also investigated.

Materials and methods

Isolation and culture condition

The studied strain was isolated in 2013 from a digestate sample obtained from the final storage tank of a biogas plant (BGP-1) located in the North of Hesse, Germany. The input material of the biogas plant was composed of 54% slurry (20:1 cattle to pig) and 46% manure (6:1 cattle to chicken) and corn and forage rye as co-substrates (Schauss et al. 2015). The biogas plant contained a continuous stirred tank reactor (CSTRs) typical for German on farm small scale systems with a two stage mesophilic digestion process (T = 44 °C). Strain KPC-SM-21^T was cultured by a selective pre-enrichment method which was applied to culture carbapenem-resistant bacteria from the collected output material. Briefly, 10 g digestate of the storage tank was incubated directly in 90 mL sterile lysogen broth (LB, Sigma-Aldrich) containing 1 mg L^{-1} meropenem (MER: $C_{17}H_{25}N_3O_5S3H_2O$, Sigma-Aldrich). After 24 h of incubation at 37 °C

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under continuous shaking at 180 rpm, 10 µL of the pre-enrichment culture was streaked on CHROMagar KPC (CHROMagar, France). The agar plate was incubated for 24 h at 37 °C. Among morphologically different colonies grown on the agar plates, one of separately lying cream-colored colony represented strain KPC-SM-21^T which was obtained as pure culture after multiple transfer steps of single colony following singular streaking on CHROMagar KPC. After purification, fresh biomass of strain KPC-SM-21^T was cultured on LB agar containing 1 mg L^{-1} meropenem and suspended in sterile Gibco newborn calf serum (NBCS, ThermoFisher Scientific) and stored at − 20 °C and − 80 °C for long-term preservation.

Phylogenetic identification

Bacterial cell lysate and 16S rRNA gene sequencing for molecular analyses was generated and performed as described by Schauss et al. (2015). Universal 16S rRNA gene targeting primers [8F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-CGGTTACCTTGTTACGACTT-3′; (Turner et al. 1999)] were used for PCR and primers 27F [5′-GAGTTTGATCMTGGCTCAG-3′; (Lane 1991)] and E786F [5′-GATTAGATACCCTGGTAG-3′; (Baker et al. 2003)] for Sanger sequencing performed at LGC Genomics (Berlin, Germany). The partial gene sequences were corrected in MEGA7 (Kumar et al. 2016) based on electropherograms and concatenated to a nearly full-length 16S rRNA gene sequence. Next related type strains were determined using the EzBioCloud 16S rRNA gene identification system (Yoon et al. 2017). The phylogenetic relationship of KPC-SM-21^T to the type strains of the genus *Acinetobacter*, including several genomic species and multiple species without validly published names, was studied based on nearly complete 16S rRNA gene sequences. 16S rRNA genes sequences of all representatives of this genus were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/) and aligned with ClustalW (Thompson et al. 1994) provided in MEGA7. The phylogenetic tree was constructed using the maximum-likelihood method (ML; Felsenstein 1981) based on the Kimura 2-parameter model (Kimura 1980). The consistency of the phylogenetic tree was investigated by 100 resamplings (bootstrap analysis; Felsenstein 1985). Moreover, phylogenetic analyses with higher resolution were performed based on protein coding sequences including the RNA polymerase β-subunit (*rpoB*) and DNA gyrase subunit B (*gyrB*) genes as described previously (Nemec et al. 2009; Krizova et al. 2014). Alignments of the nucleotide sequences of each gene were performed based on the respective correct open reading frame (ORF). Pairwise nucleotide sequence similarities were determined with the p-distance method implemented in MEGA7. Phylogenetic analyses were performed using the ML method based on the General Time Reversible (GTR; Nei and Kumar 2000) model for nucleotide and the Jones–Thornton–Taylor matrix-based (JTT; Jones et al. 1992) model for amino acid sequence based analysis. Multilocus sequence analysis (MLSA) was performed based on genes used in the multilocus sequence typing (MLST) scheme (Pasteur; https://pubmlst.org/abaumannii/) for *A. baumannii* (Diancourt et al. 2010). Partial sequences of six housekeeping genes were used for MLSA analysis. The genes code for CTP synthase (PyrG), 60-KDa chaperonin (Cpn60), citrate synthase (GltA), homologous recombination factor (RecA), 50S ribosomal protein L2 (RplB) and the beta-subunit of the RNA polymerase (RpoB), respectively. The *rpoB* gene fragment used in the MLSA approach [spanning nucleotide positions 1681–2136 of the *rpoB* gene of *A. baumannii* CIP 70.34^T (DQ207471)] was different from that applied in the *rpoB* gene based phylogeny. Full-length sequences of these housekeeping genes for type strains of *Acinetobacter* species were obtained from the NCBI database. Sequences were aligned based on the correct ORF and concatenated in the following order: *pyrG* (297 nt), *cpn60* (405 nt), *gltA* (483 nt), *recA* (361 nt), *rplB* (330 nt) and *rpoB* (456 nt) based on their respective sizes. The gene encoding for elongation factor G (FusA), mentioned in the MLST scheme, was not used in MLSA, since the amplification result of the *fusA* gene was unsatisfactory. The evolutionary history was inferred using the ML method based on the GTR model (Nei and Kumar 2000).

Genome sequencing, core genome based phylogeny and genome-wide analysis

A draft genome sequence of strain KPC-SM-21^T was generated by Illumina short read sequencing (read out 2 × 300 bp, MiSeq benchtop sequencer) followed by

sequence reconstruction using the A5-miseq assembly pipeline. Genome sequence based analyses were performed in EDGAR 2.3 (Blom et al. 2016). The genome sequence of strain KPC-SM-21^T and genome sequences of *Acinetobacter* species (validly published) type strains and strains representing distinct genomic species with provisional designation or *Acinetobacter* species without names standing in nomenclature were obtained from the NCBI database and integrated into an EDGAR project. The BLAST search of the 16S rRNA gene sequence of strain KPC-SM-21^T showed 99.7% similarity to the 16S rRNA gene of *Acinetobacter* sp. Marseille-Q1620 (LR782267.1). Therefore, the genome of *Acinetobacter* sp. Marseille-Q1620 (NZ_LR782267) was also included to determine the taxonomic position of strain KPC-SM-21^T.

The taxonomic status at the whole genome level was assessed by calculating average nucleotide identity (ANI) values. An ANI matrix was calculated in EDGAR based on the BLASTN comparison of the genome sequences as described by Goris et al. (2007). A core genome based phylogenetic analysis was calculated in EDGAR following a stepwise alignments of each core gene set using MUSCLE (implemented in EDGAR 2.3) the final alignments were concatenated to one huge alignment, which included shared genes of the genome of strain KPC-SM-21^T, the *Acinetobacter* reference genomes and the genome of *Moraxella lacunata* NBRC 102154^T (NZ_BCUK00000000) which was used as outgroup. Thereafter, a core genome based phylogenetic analysis was computed using the FastTree software (http://www.microbesonline.org/fasttree/) to generate approximately-maximum-likelihood phylogenetic trees (Price et al. 2009, 2010) implemented in EDGAR 2.3. The genome-based circular plot was generated with BioCircos (Cui et al. 2016) implemented in EDGAR 2.3. Furthermore, EDGAR 2.3 and VFDB (virulence factor database; http://www.mgc.ac.cn/VFs/) were used to identify resistance and virulence associated genes. Genomic islands (GIs) were searched with IslandViewer4 (Bertelli et al. 2017; Bertelli and Brinkman 2018). Potential phage-related genes of strain KPC-SM-21^T were identified using PHASTER (https://phaster.ca/; Zhou et al. 2011; Arndt et al. 2016).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

For MALDI-TOF MS the strain was grown on Columbia agar with 5% sheep blood (SBA; Oxoid) for 24 h. The experiment was performed as described by Eisenberg et al. (2017). Biomass was transferred to steel targets using the direct transfer protocol according to the manufacturer's instruction (MALDI Biotyper; Bruker Daltonics, Bremen, Germany). Analysis was performed on a MALDI-TOF MS Biotyper version 3.3.1.0; commercial database (DB 8468; BrukerDaltonics). The MALDI Biotyper real-time classification (RTC) software calculated obtained log score based on similarities between the observed results and stored database sets. Log scores of > 2.3 and > 2.0 were considered as species and genus level identifications, respectively. The identification was repeated three times to verify the original findings.

Fatty acid analysis

Biomass for fatty acid analysis was harvested after growth on trypticase soy agar (TS agar; Becton Dickinson GmbH) at 30 °C for 48 h (exponentially growing cells). The analysis was performed as described by Kämpfer and Kroppenstedt (1996) using the Sherlock version 2.11, TSBA40 Rev. 4.1 for identification.

Phenotypic characterization

Cell morphology and motility was observed under a Zeiss light microscope at a magnification of × 1000, using cells grown for three days at 25 °C on TS agar. Gram-staining was performed by the modified Hucker method according to Gerhardt et al. (1994). Cytochrome-c oxidase activity was tested using Bactident oxidase test strips (Merck) and catalase enzyme activity by testing formation of gas bubbles after dropping 3% (v/v) hydrogen peroxide (H_2O_2) onto overnight grown biomass on TS agar. The test of growth on different agar media and temperature-dependent growth was performed by suspending fresh biomass in 0.9% (w/v) sodium chloride (NaCl); turbidity standardized by 0.5 McFarland. The cell suspension was serially diluted up to 10^{-5} and 5 µL of each dilution were spotted on following media: TS agar, R2A agar (R2A; Oxoid), nutrient agar (NA;

Becton Dickinson), malt agar (Merck), glycine arginine agar (Gly/Arg; Oxoid), CASO agar (Carl Roth), K7 [0.1% (w/v) of yeast extract, peptone, and glucose, agar (15 g L^{-1}), pH 6.8], M65 medium (according to DSMZ), DEV agar (DEV; Merck), Luria Bertani (LB; Sigma-Aldrich), MacConkey agar (Oxoid), PYE [0.3% (w/v) yeast extract and 0.3% (w/v) casein peptone, agar (15 g L^{-1}), pH 7.2)], nutrient broth (NU; Oxoid), marine agar (MA; Becton Dickinson) and SBA, respectively. Thereafter, all plates were incubated at 28 °C and growth was analysed after 7 days. For temperature-dependent growth the serially dilutions were spotted on TS agar plates which were incubated at 4, 10, 15, 20, 25, 28, 30, 37, 45, 50, and 55 °C, respectively, as described by Pulami et al. (2020). The growth was monitored after 24 h, 48 h, 3 and 7 days of incubation. Hemolysis test was performed as previously described by Nemec et al. (2016). The physiological characterization was performed as described by Kämpfer et al. (1991). Furthermore, strain KPC-SM-21^T was tested with the API 20 NE kit (BioMérieux) following the instructions of the manufacturer.

Anaerobic growth test

The survival of strain KPC-SM-21^T and *A. baumannii* ATCC 19606^T under anaerobic conditions was investigated by taking strains pre-grown (overnight aerobically at 25 °C) on NA plates, and exposing them to anaerobic conditions using the Anaerocult A system (Merck) at the same temperature for 7 days. Thereafter, a loop of biomass was re-inoculated onto fresh NA, and growth was checked after overnight aerobic incubation at 37 °C. The ability of the strain to grow under anaerobic conditions was checked by direct exposure of streaked plates to anaerobic conditions using the Anaerocult A system at 25 °C for 7 days.

Results and discussion

Molecular and genome characteristics

The 16S rRNA gene sequence of strain KPC-SM-21^T obtained by Sanger sequencing was 1439 nucleotides in length, spanning gene termini 28 to 1468 [numbering according to the *Escherichia coli rrnB* (Brosius et al. 1978)], and initial phylogenetic assignment obtained by BLAST against the EzBioCloud database showed 97.0% similarity to *A. baumannii* ATCC 19606^T. Sequence similarities to all type strains of *Acinetobacter* species were $\leq$ 97%. This indicated that strain KPC-SM-21^T represented a novel species, because all similarity values were below that of 98.65%, which was suggested by Kim et al. (2014) as a pre-requisite threshold to delineate a prokaryotic species. The ML tree based on 16S rRNA gene sequences was based on 1223 nucleotide positions. It showed the placement of strain KPC-SM-21^T in a separate branch within the genus *Acinetobacter* without a distinct clustering to any of the other investigated strains including all type strains of the genus (Fig. 1). The *rpoB* based phylogenetic analyses included gene fragments spanning gene positions 2917–3267 (zone1) and 3322–3723 (zone2), respectively. Gene termini were given according to the gene sequence obtained from *A. baumannii* CIP 70.34^T (DQ207471, La Scola et al. 2006). The nucleotide sequence of the concatenated variable zones of the *rpoB* gene of strain KPC-SM-21^T showed highest sequence similarity to *A. gerneri* DSM 14967^T (91.1%), followed by *A. guillouiae* CIP 63.46^T (86.9%) and *A. baylyi* DSM 14961^T (86.6%); the *rpoB* sequence similarity to *A. baumannii* ATCC 19606^T was lower (82.6%). The obtained *rpoB* nucleotide sequence similarity values were below 95% to tested next related type strains of the genus *Acinetobacter*. La Scola et al. (2006) and Narciso-da-Rocha et al. (2013) have suggested that *rpoB* gene sequence similarities below 95% represent distinct *Acinetobacter* species. The ML tree based on *rpoB* nucleotide (Fig. 2) and amino acid sequences (Fig. S1) showed that strain KPC-SM-21^T formed a distinct cluster with *A. gerneri* DSM 14967^T which was supported by high bootstrap values (> 70%). GyrB based phylogenetic analysis was performed with a gene region encompassing nucleotide positions 457–1209 (numbering according to *A. baumannii* ATCC 19606^T (Genome accession number: APRG00000000, Locus tag: 911_RS22805). The *gyrB* gene sequence based analysis also showed highest nucleotide sequence similarity with *A. gerneri* DSM 14967^T (85.2%). Sequence similarities with all other tested *Acinetobacter* sp. type strains were below 83.5%. The *gyrB* nucleotide sequence based phylogenetic tree also showed a distinct cluster of KPC-SM-21^T and *A. gerneri* DSM 14967^T. However, this cluster was not supported with a high bootstrap value


A. bohemicus ANC 3994T (KF679797)
A. pakistanensis NCCP-644T (AB916465)
A. kyonggiensis KSL5401-037 (NR_116714)
A. celticus ANC 4603T (KX548338)
A. bouvetii 4B02T (AF509827
A. gyllenbergii RUH 422T (AJ293694)
A. oryzae B23 (GU954428)
A. johnsonii ATCC 17909T (Z93440)
A. proteolyticus NIPH 809T (KT997475)
A. colistiniresistens NIPH 2036T (ATGK00000000)
A. beijerinckii LUH 4759T (AJ626712)
A. genomic sp. 16 ATCC 17988 (Z93451)
A. haemolyticus DSM 6962T (X81662)
A. genomic sp. 15BJ 79 (Z93452)
A. gandensis UG 60467T (KM206131)
A. schindleri LUH 5832T (AJ278311)
A. equi 114T (KC494698)
A. kookii 11-0202T (JX137279)
A. pragensis ANC 4149T (KX014586)
A. albensis ANC 4874T (KR611798)
A. lwoffii NCTC 5866T (AIEL01000120)
A. harbinensis HITLi7T (KC843488)
A. ursingii LUH 3792T (AJ275038)
A. septicus AK001 (EF611418)
A. qingfengensis 2BJ1T (JX982123)
A. defluvii WCHA30T (KY435933)
A. piscicola LW15T (MF062566)
A. bereziniae LMG 1003T (Z93443)
A. guillouiae CIP 63.46T (X81659)
A. dispersus ANC 4105T (KT997473)
A. genomic sp. 17 942 (Z93454)
A. tjernbergiae 7N16T (AF509825)
A. parvus LUH 4616T (AJ293691)
A. tandoii 4N13T (AF509830)
A. genomic sp. 13BJ/14TU ATCC 17905 (Z93447)
A. modestus NIPH 236T (KT997474)
A. vivianii NIPH 2168T (KT997477)
A. courvalinii ANC 3623T (KT997472)
A. genomic sp. 14BJ 382 (Z93453)
A. marinus SW-3 (AY633607)
A. baumannii ATCC 19606T (ACQB01000091)
A. gerneri 9A01T (AF509829)
A. grimontii 17A04T (AF509828)
A. junii DSM 6964T (X81664)
A. plantarum THG-SQM11 (KM598254)
A. halotolerans R160T (KT032155)
A. genomic sp. 6 ATCC 17979 (Z93439)
A. puyangensis BQ4-1T (JN664255)
A. brisouii 5YN5-8T (DQ832256)
A. nosocomialis RUH 2376T (HQ180192)
A. seifertii LUH 1472T (FJ860878)
A. calcoaceticus DSM 30006T (AIEC01000170)
A. oleivorans DR1 (NR_102814)
A. genomic sp. BTW. 1 and 3 LUH 1469 (FJ860877)
A. pittii LMG 1035T (HQ180184)
A. dijkshoorniae JVAP01T (KX027435)
A. lactucae NRRL B-41902T (KU921101)
A. venetianus ATCC 31012T (AJ295007)
A. antiviralis KNF 2022 (DQ314740)
A. baylyi CIP 107474T (AF509820)
A. soli B1T (EU290155)
A. towneri DSM 14962T (APPY01000064)
A.seohaensis DSM 16313 (AY633608)
A. guangdongensis 1NM-4T (JQ608323)
A. populi PBJ7T (KM518626)
A. radioresistens DSM 6976T (X81666)
A. indicus A648T (HM047743)
A. refrigeratorensis WB1 (KF709454)
A. variabilis NIPH 2171T (KP278590)
Acinetobacter stercoris KPC-SM-21T(MT138756)
A. rudis G30T (EF204258)
A. larvae BRTC-1T (KU560468)
A. boissieri SAP 284.1T (JQ771141)
A. nectaris SAP 763.2T (JQ771132)
A. apis HYN18T (JX402203)
Moraxella lacunata CCUG 4441T (AF005160)
0.01

◀**Fig. 1** Phylogenetic placement of strain KPC-SM-21^T within the genus *Acinetobacter* based on nearly full-length 16S rRNA gene sequences. The maximum-likelihood tree was generated in MEGA7 and is based on nucleotide positions 28–1468 (according to *E. coli* numbering; Brosius et al. 1978). The respective gene sequence of the type strain of *Moraxella lacunata* was used as outgroup. Numbers at nodes represent bootstrap values (> 70%) based on 100 replications. Filled circles indicate nodes that were conserved in a tree generated with the neighbour-joining (NJ) method. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position

(Fig. S2). Similarly, the phylogeny based on amino acid sequences of *rpoB* (Fig. S1) and *gyrB* (Fig. S3) also showed the placement of strain KPC-SM-21^T in a separate branch within the genus *Acinetobacter*. The ML tree based on MLSA data placed strain KPC-SM-21^T in a separate branch beside other *Acinetobacter* sp. type strains (Fig. S4). Interspecies similarities of strain KPC-SM-21^T to other type strains was in the range of 82.7–89.4% (concatenated nucleotide sequences).

Prior to genome sequence-based analyses, the 16S rRNA gene sequence present in the genome sequence on contig OOGT01000238 (locus_tag: KPC_R004) was aligned with the Sanger sequenced 16S rRNA gene; both were identical. The draft genome sequence of strain KPC-SM-21^T (accession number OOGT01000000, Bioproject: PRJEB25537) had a total nucleotide length of 4.16 Mbp. The core genome-based phylogenetic tree (Fig. 3) showed distinct cluster of strain KPC-SM-21^T including *Acinetobacter* sp. Marseille-Q1620 with *A. gerneri* DSM 14967^T, respectively. The relationship between strain KPC-SM-21^T, *Acinetobacter* sp. Marseille-Q1620, *A. gerneri* DSM 14967^T and *A. baumannii* ATCC 19606^T at whole genome level was assessed by calculating average nucleotide identity (ANI) values in EDGAR 2.3. The ANI values were 98.3% (KPC-SM-21^T vs. *Acinetobacter* sp. Marseille-Q1620), 77.7% (KPC-SM-21^T vs. *A. gerneri* DSM 14967^T) and 73.6% (KPC-SM-21^T vs. *A. baumannii* ATCC 19606^T), respectively (Fig. S5). The core genome-based phylogeny and ANI values proved that strain KPC-SM-21^T and *Acinetobacter* sp. Marseille-Q1620 belonged to the same cluster of species and are genomically closely related. The ANI values against *A. gerneri* DSM 14967^T and *A. baumannii* ATCC 19606^T were below the threshold of ~ 95–96% proposed to discriminate between prokaryotic species (Richter and Rosselló-Móra 2009). The genomic DNA G + C content of strain KPC-SM-21^T was 37.7 mol%, which was similar to that of the two closely related type strains, 39.2 mol% for *A. baumannii* ATCC 19606^T and 37.9 mol% for *A. gerneri* DSM 14967^T, respectively.

Therefore, on the basis of 16S rRNA gene, *rpoB* comparative analysis, *gyrB* phylogeny, and MLSA, strain KPC-SM-21^T was distinct from the type strains of *Acinetobacter* species with validly published names, genomic species with provisional designation or *Acinetobacter* species without names standing in nomenclature. Notably, ANI values and core genome-based phylogeny proved the high similarity between strain KPC-SM-21^T and *Acinetobacter* sp. Marseille-Q1620 below the threshold of prokaryotic species. The strains clustered with the type strain of *A. gerneri* which is represented by two genome sequences (*A. gerneri* DSM 14967^T and *A. gerneri* CIP 63.46^T).

Assignment by MALDI-TOF and fatty acid analysis

MALDI-TOF data confirmed the genotypic identification of strain KPC-SM-21^T as novel *Acinetobacter* species. The dendrogram based on MALDI-TOF data showed a distinct clustering of strain KPC-SM-21^T (Fig. S6) among type strains of next related *Acinetobacter* species. The average log score was 1.56, which was a non-reliable score that can be explained by absence of a close relative of KPC-SM-21^T in the database used. Therefore, and in a comparison with other species from the same genus, strain KPC-SM-21^T represented a distinct species of the genus *Acinetobacter* on the basis of MALDI-TOF data.

The predominant fatty acids of KPC-SM-21^T were $C_{18:1}$ w9c (44.17%), $C_{16:0}$ (21.67%) and summed feature 3* (15.34%) (containing $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH that was not determined by Sherlock version). The fatty acid pattern is typical for the genus *Acinetobacter* (Kämpfer et al. 1993; Kim et al. 2008; Vaz-Moreira et al. 2011). The presence of minor amounts of $C_{18:3}$ ω6c (2.2%) differentiated strain KPC-SM-21^T from type strains of *A. baumannii*, *A. gerneri* and *A. guillouiae*, respectively. The details of the fatty acid profile is given in Table 1.


A. genomic sp. NIPH 3623 (APSA00000000)
A. courvalinii ANC 3623T (KT997518)
A. genomic sp. NIPH 1847 (APRR00000000)
A. vivianii NIPH 2168T (KJ124844)
A. parvus NIPH 384T (EU477107)
A. modestus NIPH 236T (KJ124832)
A. genomic sp. CIP 64.2 (APRT00000000)
A. genomic sp. 13BJ/14TU NIPH 1859 (APRZ00000000)
A. colistiniresistens NIPH 2036T (ATGK00000000)
A. genomic sp.13BJ/14TU NIPH 2036 (ATGK00000000)
A. genomic sp. NIPH 1867 (APRO00000000)
A. dispersus ANC 4105T (KT997527)
A. tjernbergiae DSM 14971T (AYEV00000000)
A. proteolyticus NIPH 809T (KJ124841)
A. gyllenbergii NIPH 2150T (EU477148)
A. genomic sp. 16 CIP 56.2 (APPH00000000)
A. genomic sp. 15BJ CIP 110321 (AQFL00000000)
A. genomic sp. ANC 3880 (APSD00000000)
A. genomic sp. 16 CIP 70.18 (APRN00000000)
A. venetianus RAG-1T (APPO00000000)
A. beijerinckii NIPH 838T (EU477124)
A. nosocomialis NIPH 2119T (APOP00000000)
A. seifertii NIPH 973T (APOO00000000)
A. baumannii ATCC 19606T (APRG00000000)
A. calcoaceticus DSM 30006T (APQI00000000)
A. genomic sp. BTW. 1 & 3 NIPH 817 (APPF01000000)
A. pittii CIP 70.29T (APQP00000000)
A. dijkshoorniae JVAP01T (LJPG01000000)
A. lactucae NRRL-B 41902T (LRPE00000000)
A. johnsonii DSM 6963T (BBTB00000000)
A. celticus ANC 4603T (MBDL00000000)
A. defluvii WCHA30T (KY435935)
A. gandensis ANC 4275T (LZDS00000000)
A. albensis ANC 4874T (FMBK00000000)
A. equi 114T (CP012808)
A. baylyi DSM 14961T (APPT00000000)
A. guillouiae CIP 63.46T (APOS00000000)
A. bereziniae LMG 1003T (APQG00000000)
A. piscicola LW15T (NIFO00000000)
A. pragensis ANC 4149T (KX014578)
A. bouvetii DSM 14964T (APQD00000000)
A. schindleri NIPH 1034T (EU477128)
A. kookii 11-0202T (JX844152)
A. lwoffii NCTC 5866T (APQS00000000)
A. genomic sp. NIPH 899 (APPE00000000)
A. variabilis NIPH 2171T (APRS00000000)
A. guangdongensis 1NM-4T (KJ701021)
A. indicus DSM 25388T (LC102682)
A. bohemicus ANC 3994T (APOH00000000)
A. harbinensis HITLi-7T (JXBK00000000)
Acinetobacter stercoris KPC-SM-21T
A. gerneri DSM 14967T (APPN00000000)
A. brisouii DSM 18516T (LC102676)
A larvae BRTC-1T (KU837228)
A. rudis G30T (ATGI00000000)
A. tandoii DSM 14970T (AQFM00000000)
A. towneri DSM 14962T (APPY00000000)
A. grimontii CIP 107470T (DQ207483)
A. junii CIP 64.5T (APPX00000000)
A. haemolyticus CIP 64.3T (APQQ00000000)
A. halotolerans R160T (KU958712)
A. genomic sp.6 NIPH 298 (APRM00000000)
A. genomic sp.6 CIP-A165 (APOK00000000)
A. ursingii NIPH 137T (EU477105)
A. soli KCTC 22184T (LC102693)
A. apis HYN18T (JX863071)
A. apis ANC 5114 (FZLN00000000)
A. nectaris SAP 763.2T (JQ771146)
A. boissieri SAP 284.1T (JQ771155)
A. radioresistens DSM 6976T (APQF00000000)
A. qingfengensis 2BJ1T (KC631629)
A. populi PBJ7T (KM518646)
A. puyangensis BQ4-1T (JX499272)
Moraxella lacunata CCUG 4441T (MXAN00000000)
0.01

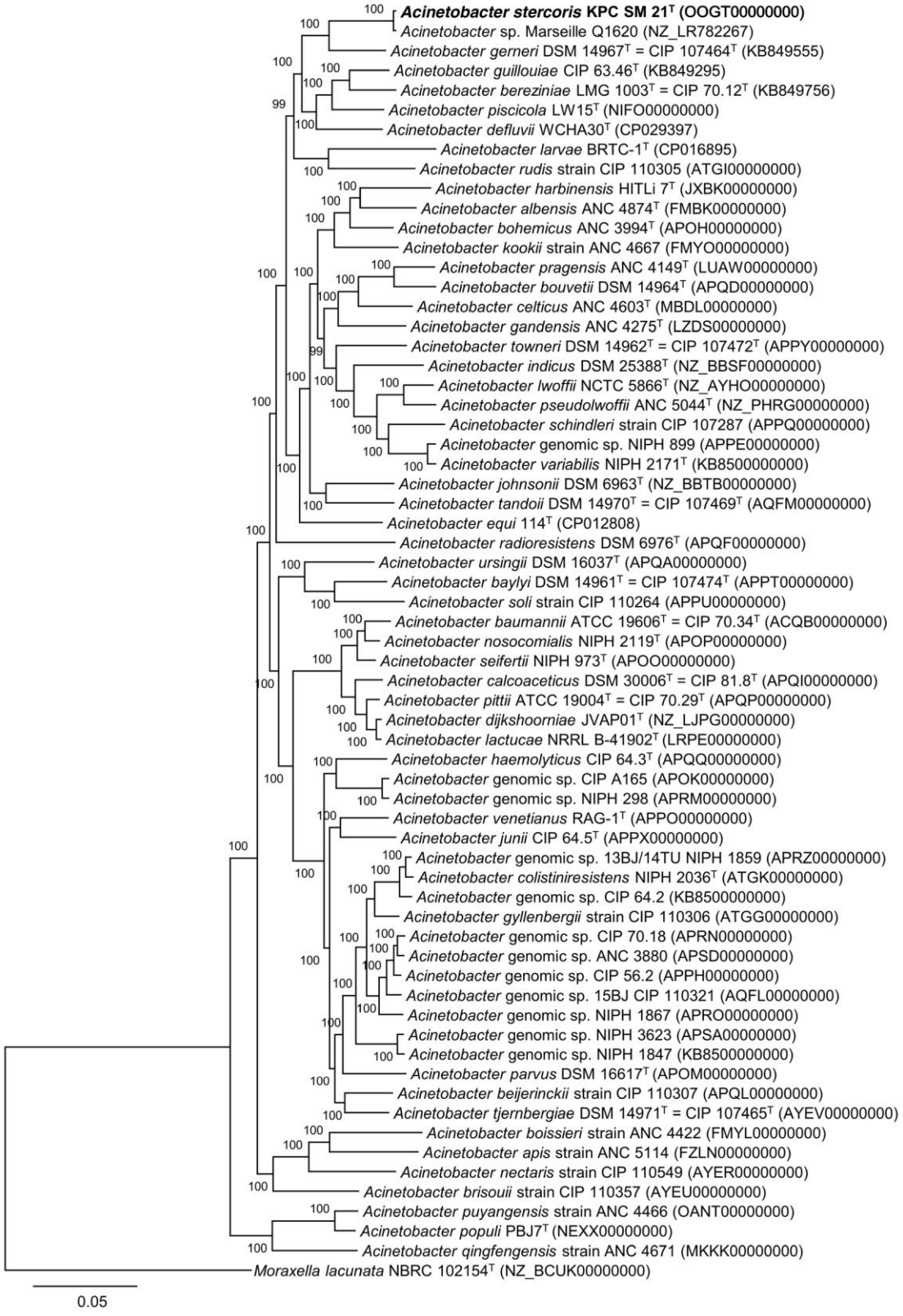
◀**Fig. 2** Phylogenetic placement of strain KPC-SM-21^T within the genus *Acinetobacter* based on nucleotide sequences of concatenated variable zones of the *rpoB* gene. The tree was calculated with the ML method based on 753 nucleotide positions in the final dataset. Numbers at nodes represent the percentage of replicate trees in which the associated taxa clustered together in bootstrap tests (100 replications). Only bootstrap values of 70% and above were shown. Filled circles indicate nodes that were also present in a tree generated with the NJ method. *Moraxella lacunata* NBRC 102154^T was used as outgroup. Bar, 0.01 substitutions per sequence position

Phenotypic characteristics

Cells of strain KPC-SM-21^T were Gram-negative, oxidase negative, catalase positive and non-motile coccobacilli as typical for members of the genus *Acinetobacter*. The optimum growth temperature was 25–37 °C; growth occurred at 45 °C and 10 °C, but not at 50 °C and 4 °C. Growth at 45 °C differentiated strain KPC-SM-21^T from type strains of *A. gerneri* (Carr et al. 2003) and *A. guillouiae* (Nemec et al. 2010). Good growth occurred at 28 °C after 24 h on TS agar, R2A, NA, malt, Gly/Arg, CASO, K7, M65, DEV, LB, PYE, NU, and SBA. Very weak growth on MA, and no growth on MacConkey agar was observed. A zone of hemolysis was not formed on SBA. The outcome of microscopy, growth at different media and range of temperature are provided in supporting information (Fig. S7, S8 and S9). Strain KPC-SM-21^T grew on a broad range of carbon sources, and showed acidification of some sugars, as α-D-glucose, α-D-lactose, L-arabinose, D-xylose, D-cellobiose, α-D-melibiose and D-mannose. However, acid production from several sugars and sugar-related compounds was not observed. Physiological tests performed with 96 wells test panel (Kämpfer et al. 1991) resulted difference in comparison with the members of the genus *Acinetobacter*. Briefly, the ability to produce acid from α-D-melibiose, and assimilation of cis-aconitate, L-aspartate, L-histidine and L-tryptophan differentiated strain KPC-SM-21^T form *A. gerneri* 9A01^T = DSM 14967^T. Formation of acid from D-glucose, D-mannose, α-D-melibiose, α-D-lactose, D-xylose and L-arabinose, and assimilation of cis-aconitate, L-phenylalanine and L-tryptophan differentiated the strain from members of *A. guillouiae* (genospecies 11). Lack of assimilation of trans-aconitate, L-arginine and L-leucine differentiated the strain from the members of *A. calcoaceticus-A. baumannii* (ACB) complex. The discriminating physiological characteristics are provided in Table 2.

Antibiotic resistance, virulence and phage associated genes

Although strain KPC-SM-21^T was isolated from non-clinical environment (output digestate of a biogas plant), it shared virulence related genes, for instance, those involved in immune evasion and cellular invasion, persistence, serum resistance, host cell lysis, inhibition of blood coagulation, in vivo survival and interspecies competition for host colonization previously reported among nosocomial *A. baumannii* strains (detailed in Table S2). The protein-protein BLAST (Blastp) of the metalloprotease (CpaA, Table S2) of strain KPC-SM-21^T shared 59% (99% query coverage) and 55.5% (99% query coverage) amino acid sequence homology with CpaA of *Acinetobacter* sp. TGL-Y2 (accession: WP_067658284) and *A. baumannii* (accession: WP_153566028). This gene was absent in *A. gerneri* DSM 14967^T (APPN00000000) which was a close relative of strain KPC-SM-21^T, and also in the clinical strains ATCC 19606^T and ATCC 17978^T of *A. baumannii* isolated during middle of the last century (Tilley et al. 2014). Strain KPC-SM-21^T harboured an intrinsic $bla_{\mathrm{OXA-like}}$ Class D beta lactamase (Locus tag: KPC_0052) without transposition of insertion sequence element upstream this gene, and the strain also lacked potent acquired antibiotic resistance genes. As indicated by Perichon et al. (2014) the class D beta lactamase genes appeared to be intrinsic to several species of the genus *Acinetobacter*. Genomic islands (GIs) searched with IslandViewer4 showed absence of GIs with acquired resistance in the genome of strain KPC-SM-21^T. Potential phage-related gene search in PHASTER showed five incomplete and fragmented phages integrated into the genome. Additionally, a phage with putative intact region (34.6 kb) available in contig NZ_OOGT01000008.1 of strain KPC-SM-21^T was found (Fig. 4 and Table S3). The intact phage region harboured segments that coded putative phage-like protein, putative head protein, putative tail protein, putative fiber protein and multiple hypothetical proteins, however lacked regions that code proteins responsible for termination, integration and lysis which are required for propagation inside the


***Acinetobacter stercoris* KPC SM 21^T (OOGT00000000)**
Acinetobacter sp. Marseille Q1620 (NZ_LR782267)
Acinetobacter gerneri DSM 14967^T = CIP 107464^T (KB849555)
Acinetobacter guillouiae CIP 63.46^T (KB849295)
Acinetobacter bereziniae LMG 1003^T = CIP 70.12^T (KB849756)
Acinetobacter piscicola LW15^T (NIFO00000000)
Acinetobacter defluvii WCHA30^T (CP029397)
Acinetobacter larvae BRTC-1^T (CP016895)
Acinetobacter rudis strain CIP 110305 (ATGI00000000)
Acinetobacter harbinensis HITLi 7^T (JXBK00000000)
Acinetobacter albensis ANC 4874^T (FMBK00000000)
Acinetobacter bohemicus ANC 3994^T (APOH00000000)
Acinetobacter kookii strain ANC 4667 (FMYO00000000)
Acinetobacter pragensis ANC 4149^T (LUAW00000000)
Acinetobacter bouvetii DSM 14964^T (APQD00000000)
Acinetobacter celticus ANC 4603^T (MBDL00000000)
Acinetobacter gandensis ANC 4275^T (LZDS00000000)
Acinetobacter towneri DSM 14962^T = CIP 107472^T (APPY00000000)
Acinetobacter indicus DSM 25388^T (NZ_BBSF00000000)
Acinetobacter lwoffii NCTC 5866^T (NZ_AYHO00000000)
Acinetobacter pseudolwoffii ANC 5044^T (NZ_PHRG00000000)
Acinetobacter schindleri strain CIP 107287 (APPQ00000000)
Acinetobacter genomic sp. NIPH 899 (APPE00000000)
Acinetobacter variabilis NIPH 2171^T (KB8500000000)
Acinetobacter johnsonii DSM 6963^T (NZ_BBTB00000000)
Acinetobacter tandoii DSM 14970^T = CIP 107469^T (AQFM00000000)
Acinetobacter equi 114^T (CP012808)
Acinetobacter radioresistens DSM 6976^T (APQF00000000)
Acinetobacter ursingii DSM 16037^T (APQA00000000)
Acinetobacter baylyi DSM 14961^T = CIP 107474^T (APPT00000000)
Acinetobacter soli strain CIP 110264 (APPU00000000)
Acinetobacter baumannii ATCC 19606^T = CIP 70.34^T (ACQB00000000)
Acinetobacter nosocomialis NIPH 2119^T (APOP00000000)
Acinetobacter seifertii NIPH 973^T (APOO00000000)
Acinetobacter calcoaceticus DSM 30006^T = CIP 81.8^T (APQI00000000)
Acinetobacter pittii ATCC 19004^T = CIP 70.29^T (APQP00000000)
Acinetobacter dijkshoorniae JVAP01^T (NZ_LJPG00000000)
Acinetobacter lactucae NRRL B-41902^T (LRPE00000000)
Acinetobacter haemolyticus CIP 64.3^T (APQQ00000000)
Acinetobacter genomic sp. CIP A165 (APOK00000000)
Acinetobacter genomic sp. NIPH 298 (APRM00000000)
Acinetobacter venetianus RAG-1^T (APPO00000000)
Acinetobacter junii CIP 64.5^T (APPX00000000)
Acinetobacter genomic sp. 13BJ/14TU NIPH 1859 (APRZ00000000)
Acinetobacter colistiniresistens NIPH 2036^T (ATGK00000000)
Acinetobacter genomic sp. CIP 64.2 (KB8500000000)
Acinetobacter gyllenbergii strain CIP 110306 (ATGG00000000)
Acinetobacter genomic sp. CIP 70.18 (APRN00000000)
Acinetobacter genomic sp. ANC 3880 (APSD00000000)
Acinetobacter genomic sp. CIP 56.2 (APPH00000000)
Acinetobacter genomic sp. 15BJ CIP 110321 (AQFL00000000)
Acinetobacter genomic sp. NIPH 1867 (APRO00000000)
Acinetobacter genomic sp. NIPH 3623 (APSA00000000)
Acinetobacter genomic sp. NIPH 1847 (KB8500000000)
Acinetobacter parvus DSM 16617^T (APOM00000000)
Acinetobacter beijerinckii strain CIP 110307 (APQL00000000)
Acinetobacter tjernbergiae DSM 14971^T = CIP 107465^T (AYEV00000000)
Acinetobacter boissieri strain ANC 4422 (FMYL00000000)
Acinetobacter apis strain ANC 5114 (FZLN00000000)
Acinetobacter nectaris strain CIP 110549 (AYER00000000)
Acinetobacter brisouii strain CIP 110357 (AYEU00000000)
Acinetobacter puyangensis strain ANC 4466 (OANT00000000)
Acinetobacter populi PBJ7^T (NEXX00000000)
Acinetobacter qingfengensis strain ANC 4671 (MKKK00000000)
Moraxella lacunata NBRC 102154^T (NZ_BCUK00000000)
0.05

◀**Fig. 3** Phylogenetic tree based on 65 genomes, built out of a core of 668 genes per genome; 43,420 in total using EDGAR 2.3 (Blom et al. 2016), applying the FastTree software (http://www.microbesonline.org/fasttree/) to generate an approximately-ML phylogenetic tree (Price et al. 2009, 2010). The values at the branches show local support values in percentage computed by FastTree using the Shimodaira–Hasegawa test. The core has 720,855 amino acid residues per genome and 46,855,575 in total. The genome of *Moraxella lacunata* NBRC 102154^T (NZ_BCUK00000000) was used to root the tree. Bar, 0.05 substitutions per amino acid sequence residue

host bacterium (Casjens 2003; Canchaya et al. 2003; Labrie et al. 2010) (Fig. S10). This intact phage region shared 51.3% of proteins (data from PHASTER) with PHAGE_Acinet_YMC11/11/R3177 (GenBank accession: NC_041866) (Table S3).

Survival in anaerobic conditions

Both strains, KPC-SM-21^T and *A. baumannii* ATCC 19606^T, failed to grow under anaerobic conditions. However, both survived in anaerobic conditions on NA plates for a week at 25 °C, and thereafter grew well in aerobic conditions at 37 °C (data not shown). Even though the genus *Acinetobacter* is generally regarded as obligate aerobe, they can survive in different anaerobic or oxygen-limited environments, including anaerobic digesters (Supaphol et al. 2011; Baek et al. 2014; Jo et al. 2015). Recently Higgins et al. (2018) reported that *Acinetobacter* spp. survived the activated anaerobic mesophilic sludge digestion in wastewater treatment plants, but were ultimately killed in alkaline lime-treated stabilized sludge. The authors illustrated in lab scale tests that *Acinetobacter* spp. were not able to grow under anaerobic conditions but survived an incubation period of four weeks under the same conditions. The digestate of the anaerobic biogas process strain KPC-SM-21^T was isolated from represented the same type of environment. Retrospective studies have shown that *Acinetobacter* spp. accumulated efficiently intracellular polyphosphates, and thereby contributing to a minor extent to the phosphate elimination in sewage treatment plants (Fuhs and Chen 1975; Deinema et al. 1980, 1985; Wentzel et al. 1986; Bark et al. 1992; Van Groenestijn et al. 1987) reported that the accumulated polyphosphates in cells act as a phosphorus reserve and might be used as energy source by enzymatic processing of the polyphosphates via combined action of polyphosphate:AMP phosphotransferase and an adenylate kinase. Comparative genome analyses performed in EDGAR revealed the presence of genes that code for these enzymes in the KPC-SM-21^T genome (Fig. S11). This process could explain the survival of aerobic organisms in anaerobic biogas plant or anaerobic sludge treatment, because the polyphosphate reservoir in *Acinetobacter* cells can be vital under anaerobic environment conditions when these strict aerobes have no other source to generate energy (Kortstee et al. 1994).

Table 1 Fatty acid composition of strain KPC-SM-21^T and selected *Acinetobacter* species

Fatty acids	1	2	3
$C_{12:0}$	3.6	4.5	4.3
$C_{12:0}$ 2-OH	4.9	2.0	4.7
$C_{12:0}$ 3-OH	3.9	3.2	6
Summed feature 2*	4.2	3.1	1.3
Summed feature 3*	15.3	17.5	15.3
$C_{16:0}$	21.7	27.8	19.6
$C_{17:1}$ ω8c	(–)	3.1	(–)
$C_{18:3}$ ω6c	2.2	(–)	(–)
$C_{18:1}$ ω7c	(–)	(–)	1
$C_{18:1}$ ω9c	44.2	38.9	41.9

The results of strain KPC-SM-21^T and *A. baumannii* ATCC 19606^T were from this study. Data for type strain of *A. gerneri* was adapted from Lee et al. (2009)

Strain: 1, KPC-SM-21^T; 2, *A. baumannii* ATCC 19606^T; 3, *A. gerneri* DSM 14967^T = KCTC 12415^T. Values are percentage of total fatty acids. Values ≤ 1 are not shown. (−), Not detected

*Summed feature 2 in the MIDI system, contained iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH

*Summed feature 3 in the MIDI system, contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH

Conclusions

The reported phenotypic, chemotaxonomic, and genotypic characteristics congruently showed that KPC-SM-21^T (genomically highly similar to *Acinetobacter* sp. Marseille-Q1620 based on ANI value and core genome-based phylogeny) represents a novel species within the genus *Acinetobacter*, which is distinct from all hitherto described members of *Acinetobacter* at the

Table 2 Physiological and metabolic characteristics of strain KPC-SM-21^T and selected species of the genus *Acinetobacter*

Characteristic	KPC-SM-21^T	ACB complex	*A. guillouiae* (genospecies 11)	*A. gerneri*
Number of strains	1	73	7	1
Acid production from				
D-Glucose	+	89	0	100
D-Cellobiose	+	89	0	100
D-Mannose	+	88	0	100
α-D-Melibiose	+	89	0	0
α-D-Lactose	+	88	0	100
D-Xylose	+	89	0	100
L-Arabinose	+	89	0	100
Assimilation of				
Adipate	+	97	100	100
Azelate	w	97	100	100
cis-Aconitate	w	95	0	0
trans-Aconitate	−	93	0	0
4-Aminobutyrate	+	100	86	100
β-Alanine	+	93	100	100
L-Arginine	−	100	0	0
L-Aspartate	+	97	100	0
Citrate	+	100	57	100
Glutarate	+	97	100	100
L-Histidine	+	100	100	0
4-Hydroxybenzoate	+	95	86	100
L-Leucine	−	99	0	0
L-Phenylalanine	+	82	0	100
L-Tryptophan	+	93	0	0
Phenylacetate	+	85	71	100

All species with validly published names include the respective type strains. The results for KPC-SM-21^T were obtained in this study, while other data were adapted from Kämpfer et al. (1993) and Carr et al. (2003). +, positive; −, negative; w, weakly positive reaction

species level of resolution. Next related species are *A. gerneri* (based on MLSA and core genome based phylogeny) and *A. baumannii* (based on 16S rRNA gene sequence identity). Although the physiological and molecular analyses revealed that *A. gerneri* CIP 107464^T = DSM 14967^T = KCTC 12415^T was next related to KPC-SM-21^T, these two taxonomic entities were unequivocally different and distant from each other at the level of species based on all characteristics studied above. The name *Acinetobacter stercoris* sp. nov. is proposed, which indicates, that the bacterium was isolated from output manure of a biogas plant. The type strain is KPC-SM-21^T (= DSM 102168^T = LMG 29413^T).

Description of *Acinetobacter stercoris* sp. nov.

Acinetobacter stercoris (ster´co.ris. L.N. stercus faeces; L. gen. n. stercoris of manure, referring to the source of the isolate).

Cells are Gram-negative, oxidase negative, catalase positive, non-hemolytic, non-motile and coccobacilli. The optimum growth temperature is 25–37 °C; growth occurs at 45 °C and 10 °C, but not at 50 °C and 4 °C. Good growth occurred at 28 °C after 24 h on TS agar, R2A, NA, malt, Gly/Arg, CASO, K7, M65, DEV, LB, PYE, NU, and SBA. Very weak growth on MA, and no growth on MacConkey agar was observed. Tests for nitrate reduction, indole production, fermentation of

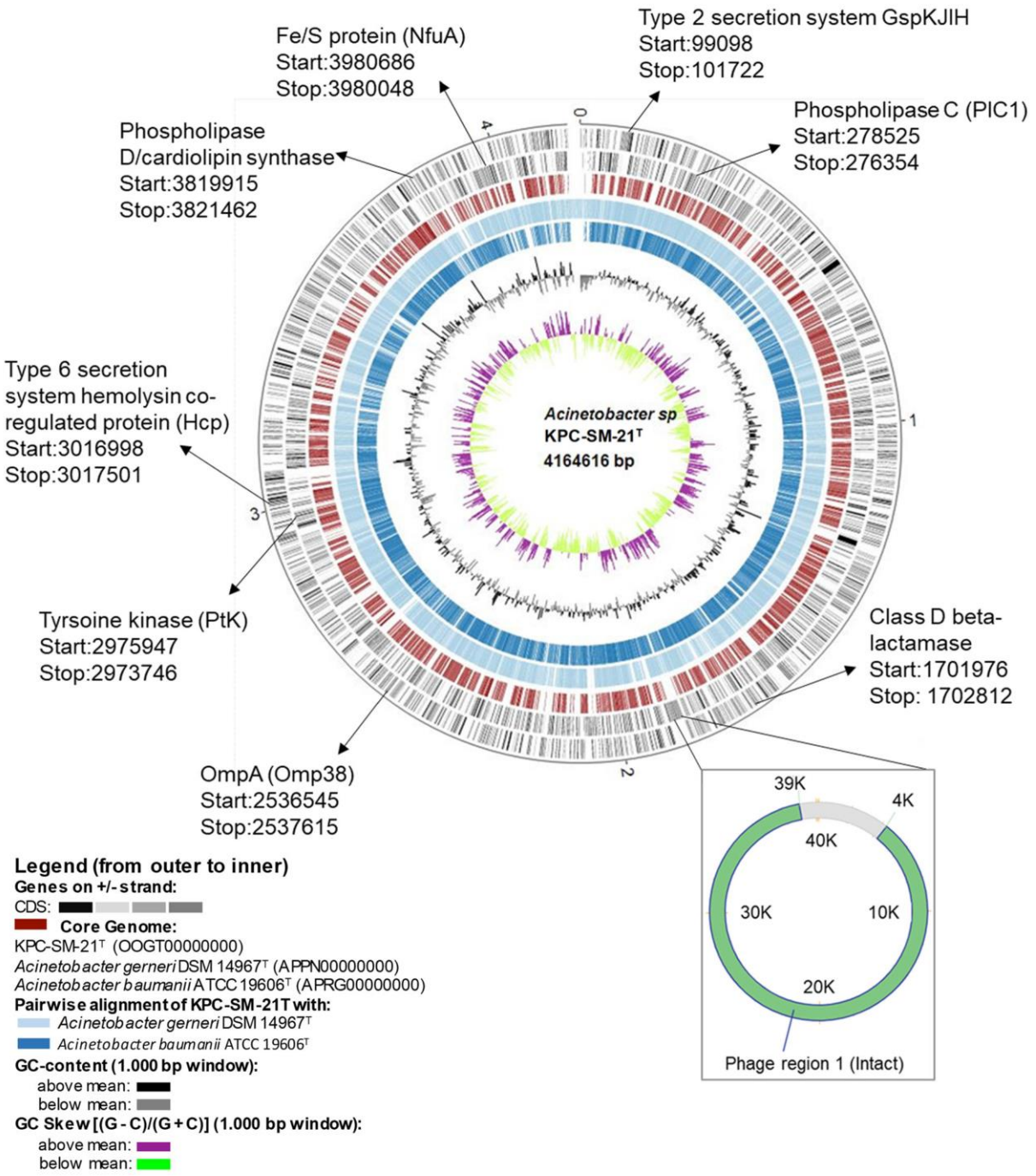


Fig. 4 A circular plot obtained from the pairwise alignment of the genomes of strain KPC-SM-21^T (size given at the center of plot), *A. gerneri* DSM 14967^T and *A. baumannii* ATCC 19606^T. The circular plot was generated with BioCircos (Cui et al. 2016) implemented in EDGAR 2.3 (Blom et al. 2016)

D-glucose, urease activity, beta-galactosidase activity, esculin and gelatin hydrolysis were negative (result from API 20 NE). No acid production from D-sucrose, D-mannitol, dulcitol, D-salicin, adonitol, i-inositol, D-sorbitol, a-D-raffinose, α-L-rhamnose, D-maltose, D-trehalose, 1-O-Methyl-D-Glucosidpyranosid,

i-erythritol, and D-arabitol. Acid was produced from α-D-glucose, α-D-lactose, L-arabinose, D-xylose, D-cellobiose, α-D-melibiose and D-mannose. Strong assimilation of N-acetyl-D-galactosamine, acetate, propionate, adipate, 4-aminobutyrate, fumarate, glutarate, DL-lactate, L-malate, 2-oxoglutarate, pyruvate, L-alanine, L-aspartate, L-histidine, L-phenylalanine, L-proline, L-tryptophan, and 4-hydroxybenzoate, and weak assimilation of D-trehalose and (DL-3-) phenylacetate was observed, respectively. No assimilation of N-acetyl-D-glucosamine, p-arbutin, D-cellobiose, D-fructose, D-galactose, D-maltose, D-mannose, α-D-melibiose, (α-) L-rhamnose, D-sucrose, adonitol, I-inositol, maltitol, D-mannitol, D-sorbitol, DL-3-hydroxybutyrate, mesaconate, L-ornithine and 3-hydroxybenzoate, N-acetyl-glucosamine, and potassium gluconate, (D-) gluconate, (α-) D-glucose, D-ribose, D-salicin, putrescine, trans-aconitate, L-leucine and L-serine. Weak assimilation of L-arabinose, D-xylose, cis-aconitate, azelate, and suberate. Strong assimilation of citrate, itaconate, β-alanine, capric acid, adipic acid, D-malate (malic acid), citrate, and phenylacetic acid. No hydrolysis of p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-phenylphosphonate, p-nitrophenyl-phosphate-disodium salt and L-proline-p-nitroanilide, p-nitrophenyl-β-D-xylopyranoside, bis-p-nitrophenyl-phosphate and L-glutamate-γ-carboxy-p-nitroanilide. However, hydrolysis of p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-phosphoryl-choline was positive. Major fatty acids were $C_{18:1}$ ω9c, $C_{16:0}$ and summed feature 3* (containing $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH that was not determined by MIDI system).

The type strain KPC-SM-21^T (= DSM 102168^T = LMG 29413^T) was isolated from the digestate of a biogas plant, located in the North of Hesse, Germany. The genomic DNA G + C content is 37.7 mol%. The NCBI/GenBank accession numbers for the whole draft genome sequence and partial 16S rRNA, *rpoB*, *gyrB* and housekeeping genes used in MLSA of KPC-SM-21^T were OOGT00000000, MT138756 and MT157622-MT157720, respectively. The complete sequences of 16S rRNA, *rpoB* and *gyrB* genes were also provided in the whole genome [16S rRNA (GenBank: OOGT01000238.1; Locus tag: KPC_R004), *rpoB* (GenBank: OOGT01000016, Locus tag: KPC_0582) and *gyrB* (GenBank: OOGT01000207.1, Locus tag: KPC_3210)].

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Authors' contribution DP and SG designed the study and wrote manuscript which was proofed by all co-authors. TS was responsible for the isolation of the strain. DP and SG performed molecular analysis; DP physiological tests. TE provided MALDI-TOF data, PK fatty acid data, JB and GW genome sequence data data, respectively. OS, JB, DP perfromed genome annotation and/or genome sequence based analyses.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical statement No specific permissions were required for these locations/activities and sampling was done with the agreement of the farmers.

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Annex

Chapter II, Supporting Information

High diversity of the emerging pathogen *Acinetobacter baumannii* and other *Acinetobacter* spp. in raw manure, Biogas Plants Digestates, and rural and urban Wastewater Treatment Plants with system specific antimicrobial resistance profiles

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Supplementray tables

Table S1. Additional characteristics of the biogas plants and WWTPs

Sampling sites	Sampling date	Sampling points and materials	Operating temperature; specific information
BGP1	July/November 2019, March/April 2020	Biogas plant input material (BGP1-I; raw cattle manure); Biogas plant output material (BGP1-O; manure digestate)	Mesophilic
BGP2	November 2019, March/April 2020	Biogas plant input material (BGP2-I; raw cattle manure); Biogas plant output material (BGP2-O; manure digestate)	Thermophilic
BGP4	October 2019	Biogas plant input material (BGP4-I; raw pig manure); Biogas plant output material (BGP4-O; manure digestate)	Mesophilic
BGP5	October 2019	Biogas plant input material (BGP5-I; mixed pig, cattle, chicken manures); Biogas plant output material (BGP5-O, manure digestate)	Mesophilic
BGP6	October 2019	Biogas plant input material (BGP6-I, raw cattle manure); Biogas plant output material (BGP6-O, manure digestate)	Mesophilic
WWTP1	November 2019, May 2020	– untreated wastewater collected after the preliminary treatment (inflow; I) – activated sludge (AS) – dewatered sludge containing primary and secondary sludge (S) – effluent of the secondary clarifier (E) – river water upstream the and downstream of the discharge of effluent (RWup and RWdown)	Rural WWTP; no clinical WW
WWTP2	August/September 2018	– untreated wastewater collected from different sewer (inflow, I) – inflow and outflow of the primary clarifier (PCi and PCo) – effluent of the secondary clarifier (E) – river water upstream and downstream the discharge of effluent (RWup and RWdown) – primary sludge (PS) – secondary sludge (SS) – sludge from the anaerobic digester containing primary and secondary sludge (ADS)	Urban WWTP; WW from veterinarian and human hospital
WWTP3	August/September 2018	– untreated wastewater collected from different sewer (inflow, I) – inflow and outflow of the primary clarifier (PCi and PCo) – effluent of the secondary clarifier (E)	Urban WWTP; WW from veterinarian and human hospital

		– river water upstream and downstream the discharge of effluent (RWup and RWdown) – primary sludge (PS) – secondary sludge (SS) – sludge from the anaerobic digester containing primary and secondary sludge (ADS) – filtrate of the anaerobic post digestor (APD-F)	

Table S2. The detail overview of cultivation conditions for WWTP and all BGPs subsamples following both direct-plating (DP) and pre-enrichment methods (PE). PE was performed in LB broth (Sigma-Aldrich, Germany) containing 1 mg L-1 meropenem [MER: C17H25N3O5S, Cayman Chemical, Germany]. CA: CHROMagarTMAcinetobacter (CHROMagar, Paris, France), CR102: MDR supplement (CHROMagar, Paris, France). I: input manure and O: digestates of biogas plants (BGPs). For WWTP1, I: influent (sewer); AS: activated sludge; S: dewatered sludge; E: effluent: RWup: upstream and RWdown: downstream of river receiving the effluent. For WWTP2 and WWTP3, I: influent sewer; PCi: inflow of primary clarifier; PCo: outflow of primary clarifier; PS: primary sludge; SS: secondary sludge; E: effluent; ADS: anaerobically digested sludge; APD-F: anerobic post digester filtrate; RWup: river water upstream and RWdown: river water downstream of the discharge of effluent.

		Direct plating		**Selective pre-enrichment** LB broth with 1 mg L^{-1} meropenem, 37°C, 24 h	
Source	**Sample/ date of sampling**	**Plating media**	**Incubation Temp**	**Streaking media after pre-enrichment**	**Incubation Temp**
BGP1	BGP1-I/ Jul 2019	CA (without cefsulodin and CR102)	37°C overnight	CA (without cefsulodin and CR102)	37°C overnight
	BGP1-O/ Jul 2019				
BGP1	BGP1-I/ Nov 2019	CA + cefsulodin 5 mg L^{-1} (with and without CR102)	37°C for 4h followed by overnight at 44°C	CA + cefsulodin 5 mg L^{-1} (with and without CR102)	37°C overnight
	BGP1-O/ Nov 2019				
	BGP1-I/ Mar 2020				
	BGP1-O/ Mar 2020				
	BGP1-I/ Apr 2020				
	BGP1-O/ Apr 2020				
BGP2	BGP2-I/ Nov 2019				
	BGP2-O/ Nov 2019				
	BGP2-I/ Mar 2020				
	BGP2-O/ Mar 2020				
	BGP2-I/ Apr 2020				
	BGP2-O/ Apr 2020				
BGP4	BGP4-I/ Oct 2019				
	BGP4-O/ Oct 2019				
BGP5	BGP5-I/ Oct 2019				
	BGP5-O/ Oct 2019				
BGP6	BGP6-I/ Oct 2019				
	BGP6-O/ Oct 2019				
WWTP1	AS/ Nov 2019				
	S/ Nov 2019				
	E/ Nov 2019				
	I/ May 2020				
	AS/ May 2020				
	E/ May 2020				
	S/ May 2020				

	RWup(W1)/ May 2020	Not analysed	Not analysed	CA + cefsulodin 5 mg L^{-1} (with and without CR102)	37°C overnight
	RWdown(W4)/ May 2020	Not analysed	Not analysed	CA + cefsulodin 5 mg L^{-1} (with and without CR102)	
WWTP2/3	I/ Aug/ Sep 2018	CA (without cefsulodin and CR102)	37°C overnight	CA (without cefsulodin and CR102)	37°C overnight
	PCi/PCo/ Aug/Sep 2018				
	PCi/PCo/ Aug/Sep 2018				
	E/ Aug/Sep 2018				
	RWup/ Aug/Sep 2018				
	RWdown/ Aug/Sep 2018				
	PS/ Aug/Sep 2018				
	SS/ Aug/Sep 2018				
	ADS/ Aug/Sep 2018				
	APD-F/ Aug/Sep 2018				

Table S3. Detail of primers used for PCRs in this study.

Targeted gene	Primer system	Primer sequence	Reference
16S RNA gene (Universal Bacteria)	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	(Lane, 1991)
	1492R	5'-GGYTACCTTGTTACGACTT-3'	
16S rRNA gene (*Acinetobacter* spp.)	Ac436f	5'-TTTAAGCGAGGAGGAGG-3'	(Vanbroekhoven et al., 2004; Zhang et al., 2009)
	Ac676r	5'-ATTCTACCATCCTCTCCC-3'	
*bla*OXA-51-like gene	OXA-51-likeF	5'-TAATGCTTTGATCGGCCTTG-3'	(Woodford et al., 2006)
	OXA-51-likeR	5'-TGGATTGCACTTCATCTTGG-3'	
*bla*OXA-51-like gene	OXA-69A	5'-CTAATAATTGATCTACTCAAG-3'	(Héritier et al., 2005; Zander et al., 2012)
	OXA-69B	5'-CCAGTGGATGGATGGATAGATTATC-3'	
IS*Aba1*	IS*Aba1*F	5'-CACGAATGCAGAAGTTG-3'	(Segal et al., 2005; Turton et al., 2006)
rpoB (*Acinetobacter* spp.)	*rpoB* Z1 Ac696F	5'-TAYCGYAAAGAYTTGAAAGAAG-3'	(La Scola et al., 2006; Nemec et al., 2009)
	rpoB Z1 Ac1093R	5'-CMACACCYTTGTTMCCRTGA-3'	
	rpoB Z2 Ac1055F	5'-GTGATAARATGGCBGGTCGT-3'	
	rpoB Z2 Ac1598R	5'-CGBGCRTGCATYTTGTCRT-3'	
bla_{KPC}	KPC-F	5'-TCGCTAAACTCGAACAGG-3'	(Monteiro et al., 2012)
	KPC-R	5'-TTACTGCCCGTTGACGCCCAATCC-3'	
bla_{OXA-48}	OXA-48-F	5'-TGTTTTTGGTGGCATCGAT-3'	
	OXA-48-R	5'-GTAAMRATGCTTGGTTCGC-3'	
bla_{VIM}	IMP-F	5'-GAGTGGCTTAATTCTCRATC-3'	
	IMP-R	5'-AACTAYCCAATAYRTAAC-3'	

Table S4. Overview of *Acinetobacter* sp. isolates isolated in this study from biogas plants (BGP1, 2 and 6) and wastewater treatment systems [WWTP1 (without hospital wastewater) and WWTP2 and 3 (with hospital wastewater)]. PE: Pre-enrichment method, DP: Direct plating method, CA: ChromAgarTMAcinetobacter (ChromAgar, France), CR102: supplement (ChromAgar, France).

	BGP1				**BGP2**			**BGP6**				**WWTP1**											**WWTP2**						**WWTP3**								
	PE, CA, Apr. 2020		PE, CA, Nov. 2019	PE, CA, Mar. 2020	PE, CA, Apr. 2020	DP, CA, Apr. 2020	PE, CA, Apr. 2020	PE, CA, Oct. 2019	DP, CA, Oct. 2019	DP, CA, Oct. 2019	PE, CA+CR102, Oct. 2019	PE, CA+CR102, Nov. 2019	PE, CA, Nov. 2019		PE, CA+CR102, Nov. 2019	DP, CA, Nov. 2019	PE, CA+CR102, May 2020	PE, CA, May 2020	PE, CA+CR102, May 2020	PE, CA, May 2020	PE, CA+CR102, May 2020	PE, CA, May 2020		PE, CA+CR102, May 2020	PE, CA, Aug. 2018	DP, CA, Aug. 2018			PE, CA, Sep. 2018	PE, CA, Aug. 2018	DP, CA, Aug. 2018	DP, CA, Sep. 2018	PE, CA. Sep. 2018			DP, CA. Aug. 2018	
Phylotype	Input cattle manure	Output digestate	Output digestate	Output digestate	Input cattle manure	Input cattle manure	Output digestate	Input cattle manure	Input cattle manure	Output digestate	Output digestate	Activated sludge	Activated sludge	Dewatered sludge	Dewatered sludge	Dewatered sludge	Inflow sewer influent	Inflow sewer influent	Activated sludge	Activated sludge	Effluent	Effluent	Upstream	Downstream	Inflow sewer influent	Inflow sewer influent	Inflow of primary clarifier	Outflow of primary clarifier	Primary sludge	Secondary sludge	Inflow of primary clarifier	Inflow of primary clarifier	Outflow of primary clarifier	Primary sludge	Secondary sludge	Anaerobically digested sludge	Anaerobic post-digestor filtrate	River downstream discharge	**Total (132 isolates)**
A-1	2				1																																**4**
A-2																												1									**1**
A-3	1				1																																**2**
A-4																										1	2					1	1	1					**6**
A-5																																					1	**1**
A-6		1			2	1	1		2	2						4		5	6	5	1	4		1	3	3	2	2	2	2		1	1			1		**52**
A-7.1								1					1		1	2	2	3	1		1	1			2	8	3		1	3		3	1		4		2		**40**
A-7.2																	1	1					1			2												**5**
A-8																										1												**1**
A-9												1		1												1		1	1	1			1						**7**
A-10																										1													**1**
A-11																															1								**1**
A-12			1					2			1			2																1					2				**9**
A-13				1							1																												**2**

Table S5. Overview of non-*Acinetobacter* sp. isolates isolated in this study from biogas plants (BGP1, 2 and 6) and rural (WWTP1) amd urban (WWTP2, 3) wastewater treatment systems. PE, Pre-enrichment method; DP, direct plating method. For biogas plants (BGP); input cattle manure (-I); digestate (-O). For WWTP1, influent sewer (WWTP1-I); effluent (WWTP1-E), dewatered sludge (WWTP1-S), activated sludge (WWTP1-AS), river water upstream the discharge of effluent (WWTP1-RWup). For WWTP2 and WWTP 3, untreated wastewater of the sewer (inflow; -I); inflow of primary clarifier (-PCi); outflow primary clarifier (-PCo); primary sludge (-PS); secondary sludge (-SS); effluent (-E); river water upstream (-RWup) and downstream (-RWdown) the discharge of effluent. The isolates were phylogenetically identified at the level of genus (>98.0% 16S rRNA gene similarity to the next related type species) based on partial 16S rRNA gene sequence (>740 nt length) using Ezbiocloud (https://www.ezbiocloud.net/identify) database

	BGP1						BGP2						BGP4		BGP5		BGP6		WWTP1							WWTP2												WWTP3												
	BGP1-0719-I	BGP1-0719-O	BGP1-1119-I	BGP1-1119-O	BGP1-0320-I	BGP1-0420-I	BGP2-1119-O	BGP2-1119-I	BGP2-0320-I	BGP2-0420-I		BGP2-0420-O	BGP4-1019-I	BGP4-1019-O	BGP5-1019-I	BGP5-1019-O	BGP6-1019-I	BGP6-1019-O	WWTP1-AS-1119	WWTP1-E-1119	WWTP1-E-0520	WWTP1-I-0520	WWTP1-S-1119	WWTP1-S-1119	WWTP1-RWup-	WWTP2-I		WWTP2-RWup		WWTP2-	WWTP2-PCi	WWTP2-PCo	WWTP2-E		WWTP2-PS		WWTP2-SS	WWTP3-RWdown		WWTP3-PCi		WWTP3-PCo		WWTP3-E		WWTP3-PS	WWTP3-SS	Number of
	PE	PE	PE	PE	PE	PE	PE	PE	PE	DP	PE	PE	PE	PE	PE	PE	DP	PE	PE	PE	PE	DP	PE	PE	DP	PE	DP	PE	PE	DP	PE	DP	PE	DP	DP	PE	DP	PE	DP	PE	DP	PE	DP	PE	DP	PE	
Genera																																																
Pseudomonas		2	3		2		2		4	1			1		1						1	2			1	13		1	7	3	1	4		2	6	3		2	7	1	4	1	1	2	8	2	1	89
Citrobacter																										2														1		1	1					5
Empedobacter								1			1		2			6											1	1		1			1														14
Raoultella																											1																				1
Chryseobacterium																		1	1	1				1					2														1				7
Elizabethkingia																													1																		1
Myroides	1		1	1	3	1	2	1	1			1	2	1		1		2		1									4				2			1				3		6						35
Stenotrophomonas														1																	1		2	1													5
Aeromonas																																	1	2					1	1	1						6
Comamonas																																		4					1		1				1		7
Lactobacillus																																									1						1
Diaphorobacter																																		2										2		4
Rhizobium																																		2										2		4
Pandoraea																																												1		1

Achromobacter																																				2													2
Burkholderia										3															1																								4
Bordetella										1																																							1
Sphingobacterium										1																																							1
Brucella																	1																																1
Cupriavidus																								1																									1
Total isolates																			52							10												74										54	190

Table S6. MLST types of *A. baumannii* isolates of this study based on Pasteur MLST scheme (https://pubmlst.org/organisms/acinetobacter-baumannii/). CC: clonal complex, IC: International clonal lineage, SLV: single locus variant, and DLV: double locus variant; n.d. (not determined). Novel STs (Pasteur scheme) are given in bold.

No	Isolates	ST (Pasteur)	*cpn60*	*fusA*	*gltA*	*pyrG*	*recA*	*rplB*	*rpoB*	CC/IC/SLV/DLV	blaOXA-51-like gene
1	SEH-3-A-V-1-A	25	3	3	2	4	7	2	4	CC25 (IC7)	OXA-64
2	SEH-3-A-V-3	2	2	2	2	2	2	2	2	CC2 (IC2)	OXA-66
3	SEH-5-A-D-1	2	2	2	2	2	2	2	2	CC2 (IC2)	OXA-66
4	SEH-5-A-D-3	**1460**	1	3	17	5	168	1	14	SLV of ST57 (CC57)	OXA-106
5	SEH-5-A-V-1	2	2	2	2	2	2	2	2	CC2 (IC2)	OXA-66
6	SEH-6-A-D-3	**1461**	3	3	16	1	3	2	4	DLV of ST300	OXA-106
7	SEH-11-A-D-8-A	**1462**	27	4	2	3	6	1	16	SLV of ST837 (CC837)	OXA-64
8	SEH-12-A-D-3	**1463**	8	1	5	3	7	1	3	DLV of ST152	OXA-64
9	SEH-13-A-D-4	427	13	3	2	3	6	1	16	n.d.	OXA-64
10	SEH-13-A-D-9	**1464**	3	4	2	2	7	2	2	Singleton	OXA-78
11	SEH-14-A-D-11	193	3	1	7	1	7	2	4	n.d.	OXA-120
12	SEH-17-A-V-5-A	203	3	4	2	2	7	1	2	n.d.	OXA-78
13	SEH-19-A-V-21	79	26	2	2	2	29	4	5	CC79 (IC5)	OXA-65
14	SEH-19-A-V-36-A	25	3	3	2	4	7	2	4	CC25 (IC7)	OXA-64
15	SEH-23-A-V-18-A	2	2	2	2	2	2	2	2	CC2 (IC2)	OXA-66
16	WWTP-AS-0520-AS-V-1	**1582**	3	2	7	2	247	1	3	SLV of ST126 (CC126)	OXA-64
17	WWTP-AS-0520-AS-V-3	**1583**	3	3	16	1	30	1	4	SLV of ST1561	OXA-106
18	WWTP-AS-0520-AS-V-5	**1584**	3	2	2	2	102	4	5	SLV of ST163 (CC163)	OXA-66
19	WWTP-AS-0520-AS-V-6	**1595**	3	3	2	4	247	2	4	SLV of ST25 (CC25)	OXA-64
20	WWTP-AS-0520-AS-V-9	1049	3	63	3	1	154	82	1	n.d.	OXA-375
21	WWTP-AS-0520-AS-V-11	**1585**	3	3	12	2	5	4	4	DLV of ST462	OXA-862
22	WWTP-AS-0520-A-V-2	**1586**	1	3	142	2	248	4	4	SLV of ST1069	OXA-688
23	WWTP-AS-0520-A-V-5	**1587**	5	12	11	2	228	9	14	SLV of ST46 (CC149)	OXA-104
24	WWTP-AS-0520-A-V-7	**1595**	3	3	2	4	247	2	4	SLV of ST25 (CC25)	OXA-64
25	WWTP-AS-0520-A-V-9	1049	3	63	3	1	154	82	1	n.d.	OXA-375
26	WWTP-AS-0520-A-V-12	**1588**	1	20	142	2	248	132	4	DLV of ST1586	OXA-688
27	WWTP-E-0520-AS-V-3	**1583**	3	3	16	1	30	1	4	SLV of ST1561	OXA-106
28	WWTP-E-0520-A-V-3	**1589**	3	5	5	1	247	1	4	SLV of ST132 (CC132)	OXA-120
29	WWTP-E-0520-A-V-6	**1590**	3	2	148	5	137	8	14	SLV of ST1066	OXA-391
30	WWTP-E-0520-A-V-7	182	6	3	8	2	30	5	4	n.d.	OXA-51
31	WWTP-I-0520-AS-V-2	367	1	2	14	3	12	1	2	n.d.	OXA-65
32	WWTP-I-0520-AS-V-5	**1591**	3	3	16	1	13	1	5	SLV of ST1303 (CC1303)	OXA-431
33	WWTP-I-0520-AS-V-7	**1583**	3	3	16	1	30	1	4	SLV of ST1561	OXA-106

34	WWTP-I-0520-A-V-2	**1591**	3	3	16	1	13	1	5	SLV of ST1303 (CC1303)	OXA-431
35	WWTP-S-1119-A-D-2a	**1592**	8	4	5	3	30	1	2	Singleton	OXA-64
36	WWTP-S-1119-A-D-3	**1593**	1	4	2	1	249	2	2	SLV of ST139 (CC139)	OXA-117
37	WWTP-W4-0520-AS-V-1	1049	3	63	3	1	154	82	1		OXA-375
38	WWTP-S-1119-A-D-4	**1594**	39	2	2	2	137	27	4	SLV of ST150	OXA-121
39	WWTP-S-1119-A-D-6	**1595**	3	3	2	4	247	2	4	SLV of ST25 (CC25)	OXA-64
40	WWTP-I-0520-A-V-13a	**1596**	1	49	7	3	12	133	5	SLV of ST1443	OXA-69
41	WWTP-E-0520-A-V-2a	221	3	1	2	1	18	1	48	n.d.	OXA-65
42	BGP 1-0420-O-A-V-2	1027	27	3	6	2	29	4	4	n.d.	OXA-104
43	BGP 2-0420-I-A-V-2	**1600**	12	1	2	2	29	1	5	SLV of ST309 (CC309)	OXA-51
44	BGP 2-0420-I-A-V-7	**1599**	157	3	2	2	5	4	14	SLV of ST462 (CC462)	OXA-51
45	BGP 2-0420-O-A-V-5	**1600**	12	1	2	2	29	1	5	SLV of ST309 (CC309)	OXA-51
46	BGP6-1019-O-A-D-1	**1601**	27	1	2	5	29	1	2	Singleton	OXA-91
47	BGP6-1019-O-A-D-2	917	3	2	2	30	30	74	3	n.d.	OXA-385
48	BGP6-1019-I-A-D-1	917	3	2	2	30	30	74	3	n.d.	OXA-385
49	BGP6-1019-I-A-D-2	**1601**	27	1	2	5	29	1	2	Singleton	OXA-91

Table S7. Antimicrobial susceptibility patterns of *Acinetobacter* isolates from this study tested with the Micronaut-S MDR MRGN-Screening 3 system (Merlin, Bornheim-Hersel) according to the CLSI guideline M100-ED30 (Clinical and Laboratory Standards Institute 2020). S, susceptible (green); I, intermediate (yellow); R, resistant (red) was perfromed accordin to EUCAST (Bacteria Version 11, 1 Jan. 2021) and CLSI clinical breakpoints. MIC values (mg/L concentration of antibiotics) are given for antibiotics lacking clinical breakpoints and adequate range of concentration in the Micronaut-S MDR MRGN-Screening 3 system. *A. baumannii* isolates are indicated by bold font. Non-baumannii *Acinteobacter* sp. isolates are given by normal font.

No.	*Acinetobacter* isolates	LEV	TGC	CIP	COL	FOS	CMP	T/S	TMO	CTX	AMK	CAZ	CZB	CTA	IMP	CAA	MER	MEE	MEB	PIT	PIP
1	**WWTP-AS-0520-AS-V-1**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	2/4	S	0,5	1	S	S
2	**WWTP-AS-0520-AS-V-3**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	1	<1/4	S	2/4	S	<0,25	1	S	S
3	**WWTP-AS-0520-AS-V-5**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
4	**WWTP-AS-0520-AS-V-6**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	2/4	S	<0,25	2	S	S
5	**WWTP-AS-0520-AS-V-9**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	16	<1/4	S	4/4	S	1	16	S	S
6	**WWTP-AS-0520-AS-V-11**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	4	2/4	S	8/4	S	0,5	2	S	S
7	**WWTP-AS-0520-A-V-2**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	8/4	S	0,5	2	S	S
8	**WWTP-AS-0520-A-V-5**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	2	S	S
9	**WWTP-AS-0520-A-V-7**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	2	S	S
10	**WWTP-AS-0520-A-V-9**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	2/4	S	<0,25	1	S	S
11	**WWTP-AS-0520-A-V-12**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
12	**WWTP-E-0520-AS-V-3**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	1	<1/4	S	2/4	S	<0,25	0,5	S	S
13	**WWTP-E-0520-A-V-3**	S	<0.25	S	S	>64	>16	S	128	2	S	S	2	<1/4	S	2/4	S	<0,25	1	S	S
14	**WWTP-E-0520-A-V-6**	S	<0.25	S	4[R]	>64	>16	S	128	>2	S	S	4	<1/4	S	4/4	S	0,5	2	S	S
15	**WWTP-E-0520-A-V-7**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	1	8	S	S
16	**WWTP-I-0520-AS-V-2**	S	<0.25	S	S	64	<8	S	128	>2	S	S	1	<1/4	S	2/4	S	<0,25	0,5	S	S
17	**WWTP-I-0520-AS-V-5**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	2/4	S	0,5	2	S	S
18	**WWTP-I-0520-AS-V-7**	S	<0.25	S	S	>64	>16	S	<32	2	S	S	1	<1/4	S	2/4	S	<0,25	1	S	S
19	**WWTP-I-0520-A-V-2**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
20	**WWTP-S-1119-A-D-2a**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	4	2/4	S	2/4	S	<0,25	2	S	S
21	**WWTP-S-1119-A-D-3**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	2	S	S
22	**WWTP-W4-0520-AS-V-1**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
23	**WWTP-S-1119-A-D-4**	S	<0.25	S	S	>64	>16	S	>128	>2	S	16[I]	4	<1/4	S	8/4	S	0,5	2	S	32/4[I]
24	**WWTP-S-1119-A-D-6**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	2/4	S	0,25	2	S	S
25	**WWTP-I-0520-A-V-13a**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	4	<1/4	S	8/4	S	1	2	S	S
26	**WWTP-E-0520-A-V-2a**	S	0.5	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	8/4	S	1	2	S	S
27	**BGP 1-0420-O-A-V-2**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
28	**BGP 2-0420-I-A-V-2**	S	<0.25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
29	**BGP 2-0420-I-A-V-7**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	1	<1/4	S	4/4	S	0,5	1	S	S
30	**BGP 2-0420-O-A-V-5**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	2	S	S
31	**BGP6-1019-O-A-D-1**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	8[R]	4/4	S	0,5	2	S	S
32	**BGP6-1019-O-A-D-2**	S	0.5	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
33	**BGP6-1019-I-A-D-1**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	0,5	2	S	S
34	**BGP6-1019-I-A-D-2**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
35	**BGP 2-0420-I-A-D-1**	S	<0.25	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	8/4	S	0,5	4	S	S
36	**SEH-3-A-V-1-A**	2[R]	<0,25	>2[R]	S	>64	>16	>4/76[R]	>128	>2	>32[R]	>128[R]	>32	>8/4	>8[R]	2/4	16[R]	2	>32	64/4[I]	32/4[I]
37	**SEH-3-A-V-3**	>2[R]	<0,25	>2[R]	4[R]	>64	>16	>4/76[R]	>128	>2	S	128[R]	16	8/4	>8[R]	8/4	32[R]	1	32	64/4[I]	32/4[I]
38	**SEH-5-A-D-1**	>2[R]	<0,25	>2[R]	4[R]	>64	>16	>4/76[R]	>128	>2	S	64[R]	16	>8/4	>8[R]	4/4	32[R]	2	>32	64/4[I]	32/4[I]
39	**SEH-5-A-D-3**	S	<0,25	S	S	>64	>16	S	128	>2	S	S	16	<1/4	S	<1/4	S	<0,25	1	S	S

40	**SEH-5-A-V-1**	>2[R]	<0,25	>2[R]	S	>64	>16	>4/76[R]	>128	>2	S	64[R]	16	8/4	>8[R]	4/4	32[R]	2	>32	64/4[I]	32/4[I]
41	**SEH-6-A-D-3**	S	<0,25	S	S	>64	>16	S	128	2	S	S	1	<1/4	S	<1/4	S	<0,25	1	S	S
42	**SEH-11-A-D-3**	S	<0,25	S	S	>64	>16	S	<32	<1	S	S	0,5	<1/4	S	<1/4	S	<0,25	<0,25	S	S
43	**SEH-11-A-D-8-A**	S	<0,25	2[R]	>8[R]	>64	<8	>4/76[R]	128	>2	>32[R]	64[R]	16	>8/4	>8[R]	>16/4	32[R]	<0,25	>32	64/4[I]	32/4[I]
44	**SEH-12-A-D-3**	>2[R]	>4	>2[R]	>8[R]	>64	>16	>4/76[R]	>128	>2	>32[R]	32[R]	32	>8/4	S	>16/4	32[R]	4	32	64/4[I]	32/4[I]
45	**SEH-13-A-D-4**	S	<0,25	S	S	>64	>16	S	<32	2	S	S	0,5	<1/4	S	<1/4	S	<0,25	0,5	S	S
46	**SEH-13-A-D-9**	S	<0,25	S	S	>64	>16	S	<32	<1	S	S	0,5	<1/4	S	<1/4	S	<0,25	0,5	S	S
47	**SEH-14-A-D-11**	S	<0,25	S	S	64	>16	S	<32	<1	S	S	0,5	<1/4	S	<1/4	S	<0,25	0,5	S	S
48	**SEH-17-A-V-4**	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
49	**SEH-17-A-V-5-A**	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
50	**SEH-19-A-V-21**	S	<0,25	S	S	>64	>16	S	128	>2	S	S	4	<1/4	S	2/4	S	<0,25	1	S	S
51	**SEH-19-A-V-36-A**	2[R]	<0,25	>2[R]	S	>64	>16	>4/76[R]	128	>2	>32[R]	S	2	<1/4	>8[R]	<1/4	32[R]	2	32	64/4[I]	32/4[I]
52	**SEH-23-A-V-18-A**	>2[R]	<0,25	>2[R]	S	>64	>16	>4/76[R]	>128	>2	S	S	32	>8/4	>8[R]	4/4	32[R]	2	32	64/4[I]	32/4[I]
53	SEH-1-A-D-2	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	4	4/4	S	4/4	S	<0,25	0,5	S	S
54	SEH-2-A-D-2	S	1	S	S	>64	>16	S	<32	>2	S	S	8	<1/4	S	2/4	S	<0,25	0,5	S	S
55	SEH-3-A-V-2-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	8	2/4	>8[R]	4/4	32[R]	<0,25	32	S	S
56	SEH-3-A-D-5	S	1	S	S	>64	>16	S	>128	>2	S	S	4	<1/4	S	4/4	S	<0,25	2	S	S
57	SEH-4-A-D-1	S	0,5	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	2/4	S	<0,25	0,5	S	S
58	SEH-4-A-D-6	S	0,5	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	4/4	S	<0,25	1	S	S
59	SEH-4-A-V-1	S	<0,25	S	S	>64	>16	S	>128	>2	S	16[I]	8	2/4	8[R]	8/4	S	<0,25	16	64/4[I]	32/4[I]
60	SEH-5-A-D-5	S	1	S	S	>64	>16	S	>128	>2	S	S	4	<1/4	S	4/4	S	<0,25	4	S	S
61	SEH-5-A-D-6	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	2	<1/4	S	2/4	S	<0,25	0,5	S	S
62	SEH-6-A-D-5	S	1	S	S	>64	>16	S	128	>2	S	S	8	4/4	S	1/4	S	<0,25	0,5	S	S
63	SEH-11-A-D-9	S	1	S	S	>64	<8	S	>128	>2	S	S	4	<1/4	S	4/4	S	<0,25	4	S	S
64	SEH-11-A-D-2	S	0,5	S	S	>64	>16	S	128	>2	S	S	2	<1/4	S	<1/4	S	<0,25	1	S	S
65	SEH-11-A-D-4	S	1	S	S	>64	>16	S	>128	>2	S	S	4	<1/4	S	4/4	S	<0,25	1	S	S
66	SEH-12-A-D-1	2[R]	1	>2[R]	S	>64	>16	>4/76[R]	>128	>2	S	S	4	<1/4	S	4/4	S	<0,25	8	S	S
67	SEH-12-A-D-5	2[R]	4	>2[R]	>8[R]	>64	>16	S	128	>2	>32[R]	128[R]	16	>8/4	>8[R]	16/4	32[R]	<0,25	>32	32/4[I]	32/4[I]
68	SEH-12-A-D-2	S	0,5	S	S	>64	16	>4/76[R]	128	>2	S	S	4	<1/4	S	2/4	S	<0,25	1	S	S
69	SEH-14-A-D-3	S	2	>2[R]	S	>64	>16	S	128	>2	S	S	2	>2/4	S	4/4	S	<0,25	1	S	S
70	SEH-17-A-V-8-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	4	<1/4	S	8/4	S	<0,25	4	S	S
71	SEH-19-A-V-29-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	8	<1/4	S	4/4	S	<0,25	2	S	S
72	SEH-19-A-V-10	S	<0,25	S	S	>64	>16	S	>128	>2	S	16[I]	16	4/4	8[R]	8/4	S	<0,25	32	S	S
73	SEH-19-A-V-5	S	<0,25	S	S	>64	>16	S	>128	>2	S	16[I]	8	2/4	S	4/4	16[R]	<0,25	32	32/4[I]	32/4[I]
74	SEH-20-A-V-12-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	8	<1/4	S	8/4	S	<0,25	8	S	S
75	SEH-20-A-V-7-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	8	2/4	>8[R]	4/4	64[R]	<0,25	>32	32/4[I]	32/4[I]
76	SEH-20-A-V-6-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	16[I]	8	2/4	>8[R]	4/4	32[R]	<0,25	>32	S	S
77	SEH-20-A-V-1-B	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	8	2/4	S	16/4	S	<0,25	4	S	S
78	SEH-23-A-V-1-A	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	4	2/4	S	4/4	S	<0,25	8	S	S
79	SEH-23-A-V-1-B	S	<0,25	S	S	>64	>16	S	>128	>2	S	S	16	<1/4	S	4/4	S	<0,25	8	S	S

Supplementary figures

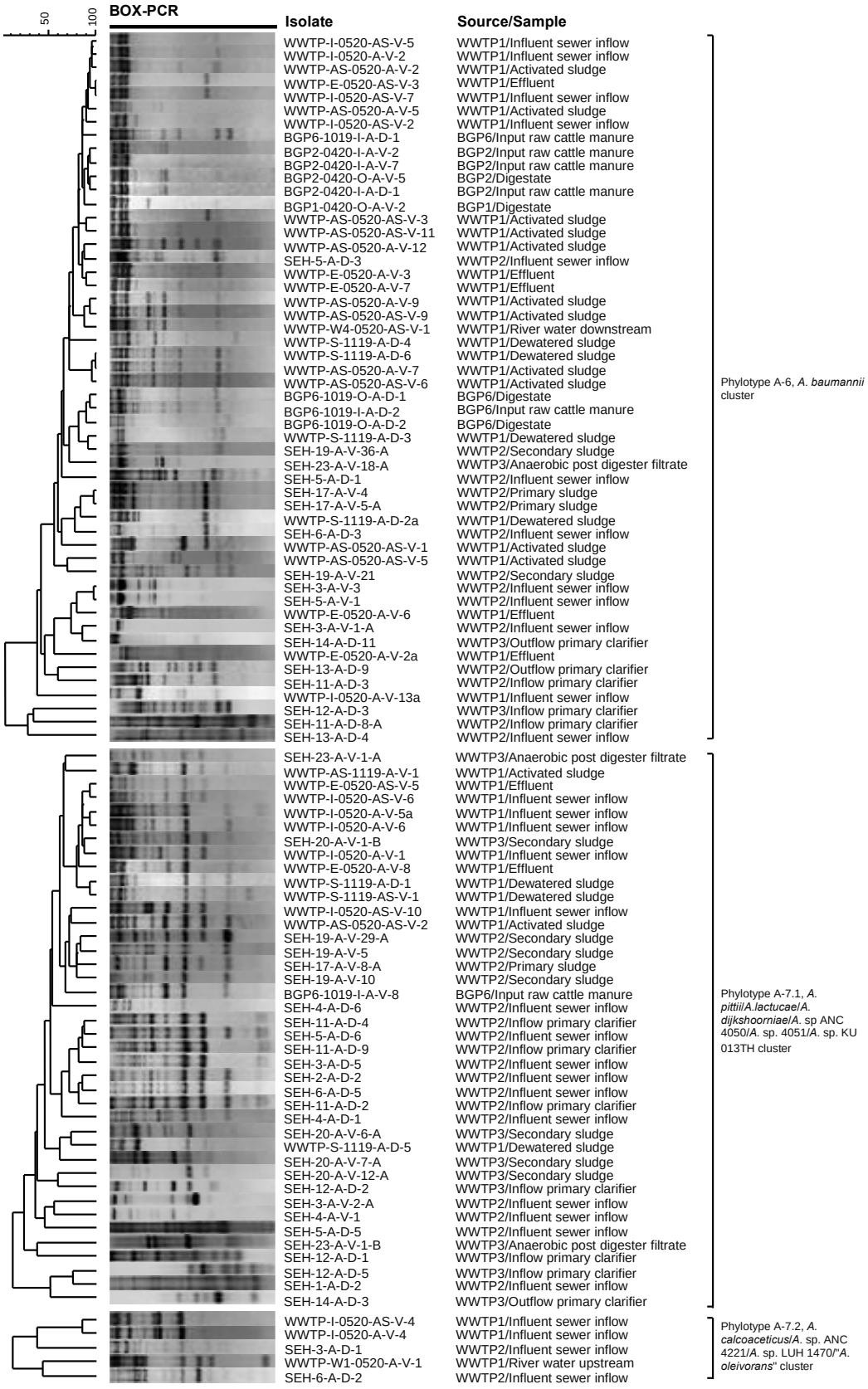


Figure S1. Genotyping of ACB complex isolates based on genomic fingerprinting patterns obtained by BOX-PCR. Cluster analysis was performed in Bionumeric version

8 using UPGMA clustering based on a pairwise distance matrix generated with the Pearson correlation coefficient. BGPs: Biogas plants; WWTPs: Wastewater treatment plants. Phylotypes were defined based on 16S rRNA and *rpoB* gene sequence analyses.

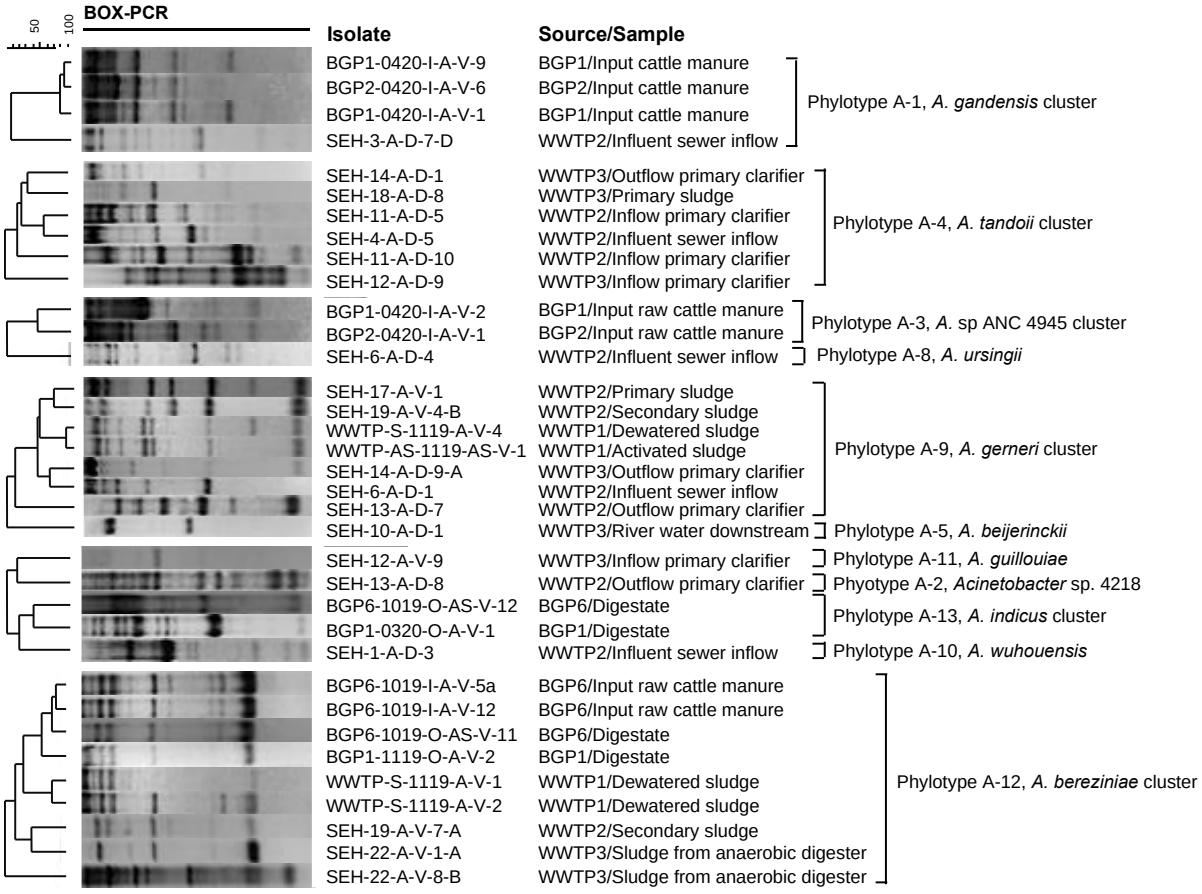


Figure S2. Cluster analyses of non-ACB complex *Acinetobacter* sp. isolates based on genomic fingerprint patterns obtained by BOX PCR. Cluster analysis was performed in Bionumeric version 8 using UPGMA clustering based on a pairwise distance matrix generated with the Pearson correlation coefficient.

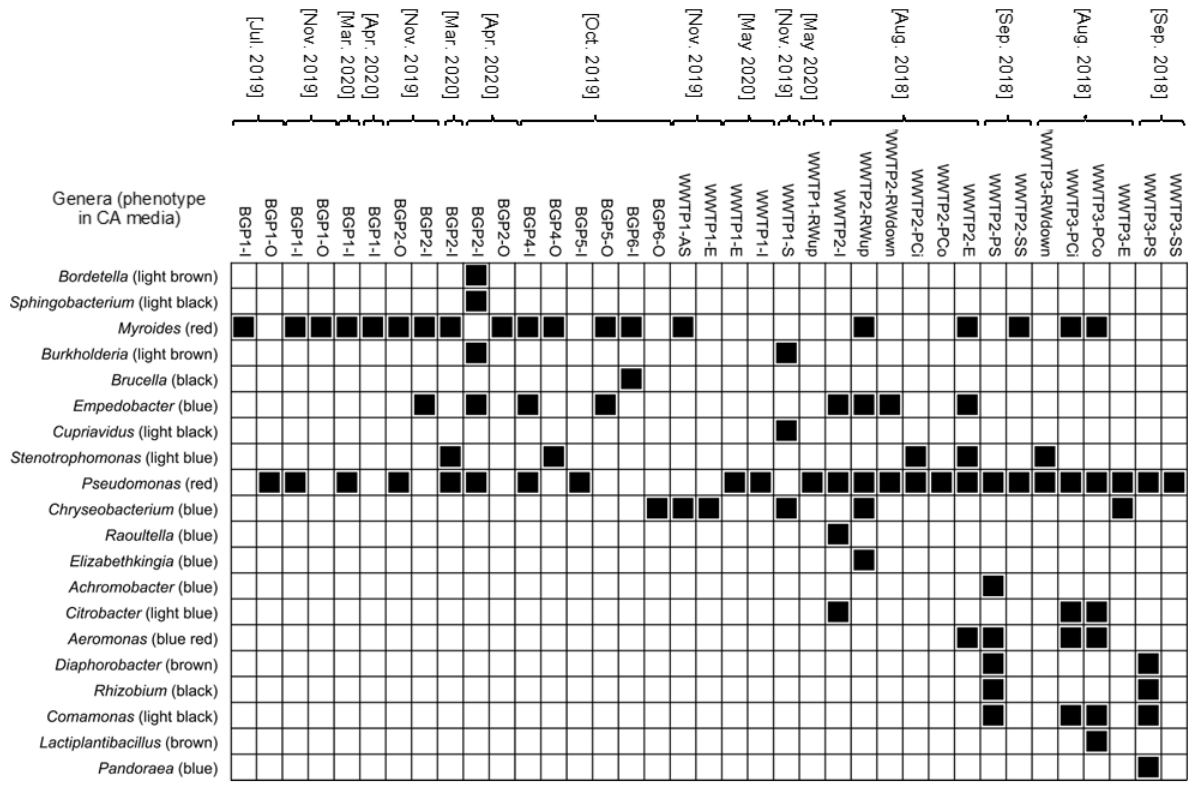


Figure S3. Serriation analysis based on the absence-presence (0/1) matrix illustrating the presence of non-*Acinetobacter* sp. isolates (non target bacteria) in different sampling points of biogas plants (BGP1, 2, 4, 5 and 6) and wastewater treatment systems (WWTP1: without hospital wastewater, WWTP2 and 3: receiving hospital wastewater). Biogas plants, I: input (raw manure), O: Output (digested manure). WWTP1, AS: activated sludge; S: dewatered sludge; I: influent (untreated wastewater); E: effluent (treated wastewater), and RWup: river water upstream. For WWTP2 and 3, I: influent (sewer, untreated wastewater); PCi: inflow of the primary clarifier; PCo: outflow of the primary clarifier; PS: primary sludge; SS: secondary sludge; RWup: river water upstream; RWdown: river water downstream, and E: effluent (treated wastewater). CA: CHROMagar^{TM}Acinetobacter agar plates. Isolates were assigned to the level of genera by phylogenetic identification based on partial 16S rRNA gene sequences.

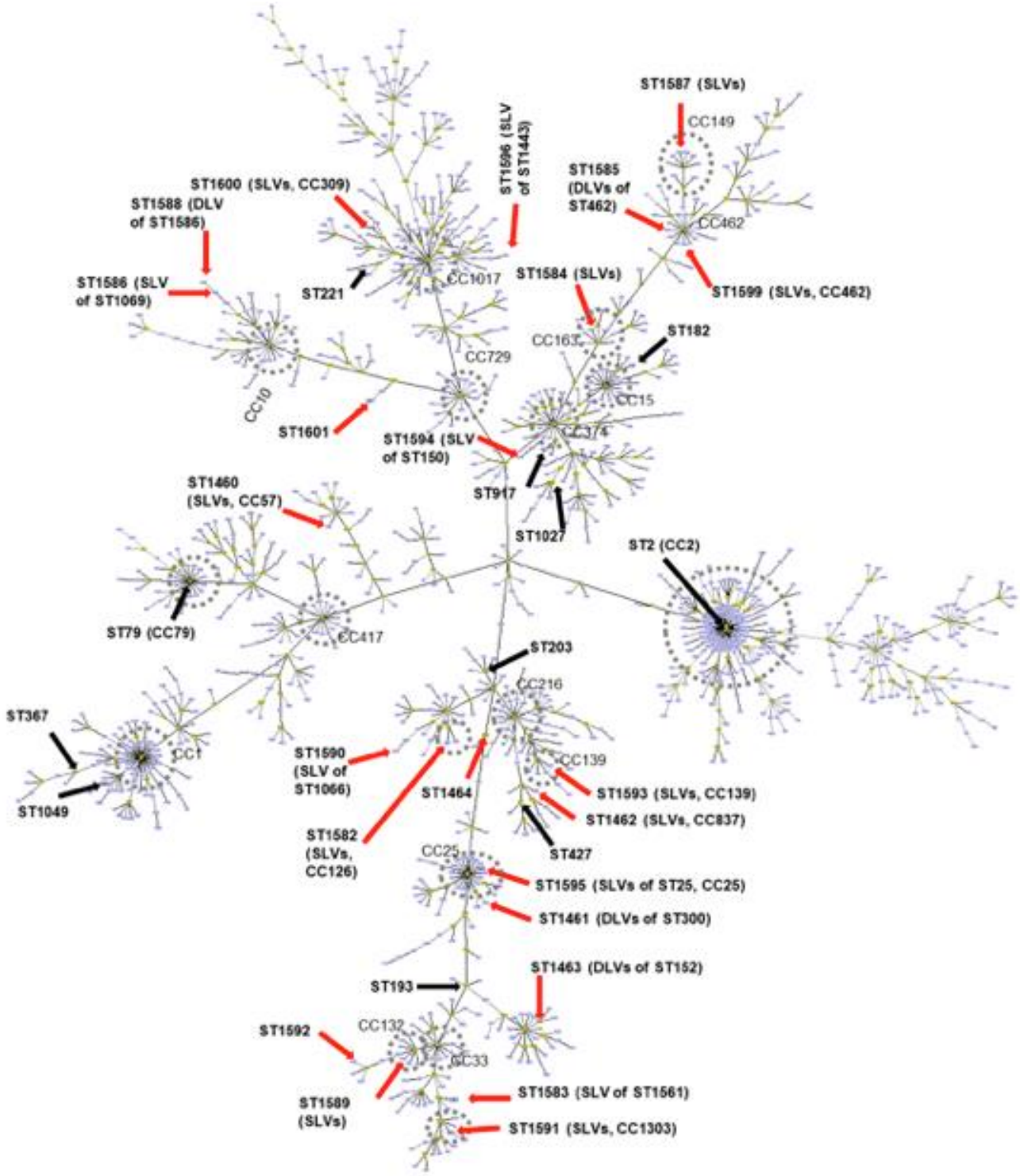
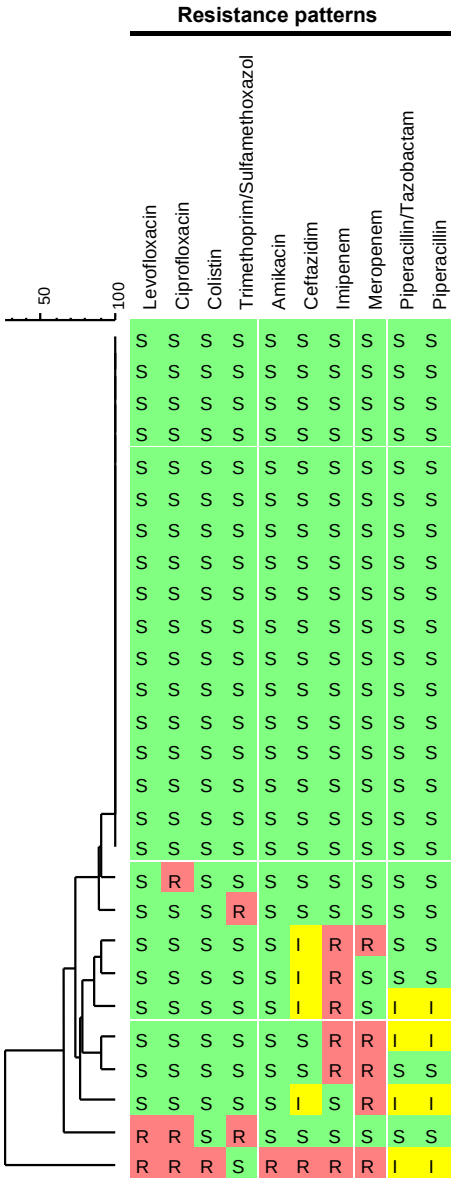


Figure S4. Combined population structure analysis based on STs of this study defined by the Pasteur MLST database of *A. baumannii.* Analysis was performed by the goeBURST algorithm in phyloviz version 2.0. Novel STs and previously defined STs found in this study were represented by red and black arrows. Founder STs were colored in light green and remaining STs in light blue. Numbers along the line indicated the variation among the seven loci between two connected strains. Grey circle

indicated major clonal complexes (CC). SLVs: Single locus variants. DLVs: Double locus variants.



Isolates	EzBioCloud database based partial 16s rRNA gene similarity	Isolation method /medium	Isolation point	WWTPs
SEH-2-A-D-2	99.7% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-11-A-D-9	100% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP2
SEH-6-A-D-5	100% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-5-A-D-5	99.9% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-5-A-D-6	99.9% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-1-A-D-2	99.2% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-4-A-D-6	100% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-11-A-D-2	99.6% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP2
SEH-11-A-D-4	100% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP2
SEH-4-A-D-1	99.8% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-3-A-D-5	100% to *A. pittii* CIP 70.29T	DP/CA	Inflow sewer	WWTP2
SEH-20-A-V-12-A	99.3% to *A.* sp. ANC 4050	PE/CA	SS	WWTP3
SEH-20-A-V-1-B	99.9% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP3
SEH-19-A-V-29-A	99.9% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP2
SEH-23-A-V-1-A	100% to *A. pittii* CIP 70.29T	PE/CA	APD-F	WWTP3
SEH-23-A-V-1-B	100% to *A. pittii* CIP 70.29T	PE/CA	APD-F	WWTP3
SEH-17-A-V-8-A	99.7% to *A. pittii* CIP 70.29T	PE/CA	PS	WWTP2
SEH-14-A-D-3	99.4% to *A. pittii* CIP 70.29T	DP/CA	PCo	WWTP3
SEH-12-A-D-2	100% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP3
SEH-20-A-V-6-A	99.9% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP3
SEH-19-A-V-10	99.8% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP2
SEH-4-A-V-1	99.8% to *A. pittii* CIP 70.29T	PE/CA	Inflow sewer	WWTP2
SEH-20-A-V-7-A	99.5% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP3
SEH-3-A-V-2-A	99.6% to *A.* sp. ANC 4050	PE/CA	Inflow sewer	WWTP2
SEH-19-A-V-5	99.7% to *A. pittii* CIP 70.29T	PE/CA	SS	WWTP2
SEH-12-A-D-1	99.8% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP3
SEH-12-A-D-5	99.7% to *A. pittii* CIP 70.29T	DP/CA	PCi	WWTP3

Figure S5. Dendrogram based on UPGMA hierarchical clustering method calculated in Bionumerics v.8 (Applied Maths, Belgium). All *Acinetobacter* sp. isolates were typically clustered based on their resistance categorical values [absent value=0, susceptible (S)= 1, intermediate (I)= 2, and resistant (R)= 3] using a categorical coefficient, which treats different values as different states. Antibiotic susceptibility profile of each isolate was converted into resistance categorical value in bionumerics v.8. The colors in the comparison window correspond to the color of each antibiotic category (S, I, R). Novel STs (Pasteur MLST) were highlighted in bold. PE: Pre-enrichment. DP: Direct plating. CA: ChromAgar^{TM}Acinetobacter media. CR102: ChromAgar MDR supplement. WWTPs: Wastewater treatment plants. BGPs: Biogas plants. PCi: Input primary clarifier. PCo: Output primary clarifier. PS: Primary sludge.

SS: Secondary sludge. APD-F: Anaerobic post digestor filtrate. Not determined: n.d. Parital 16S rRNA gene based similarity in parentheses are related to isolates identified based on identical fingerprinting patterns (BOX-PCR).

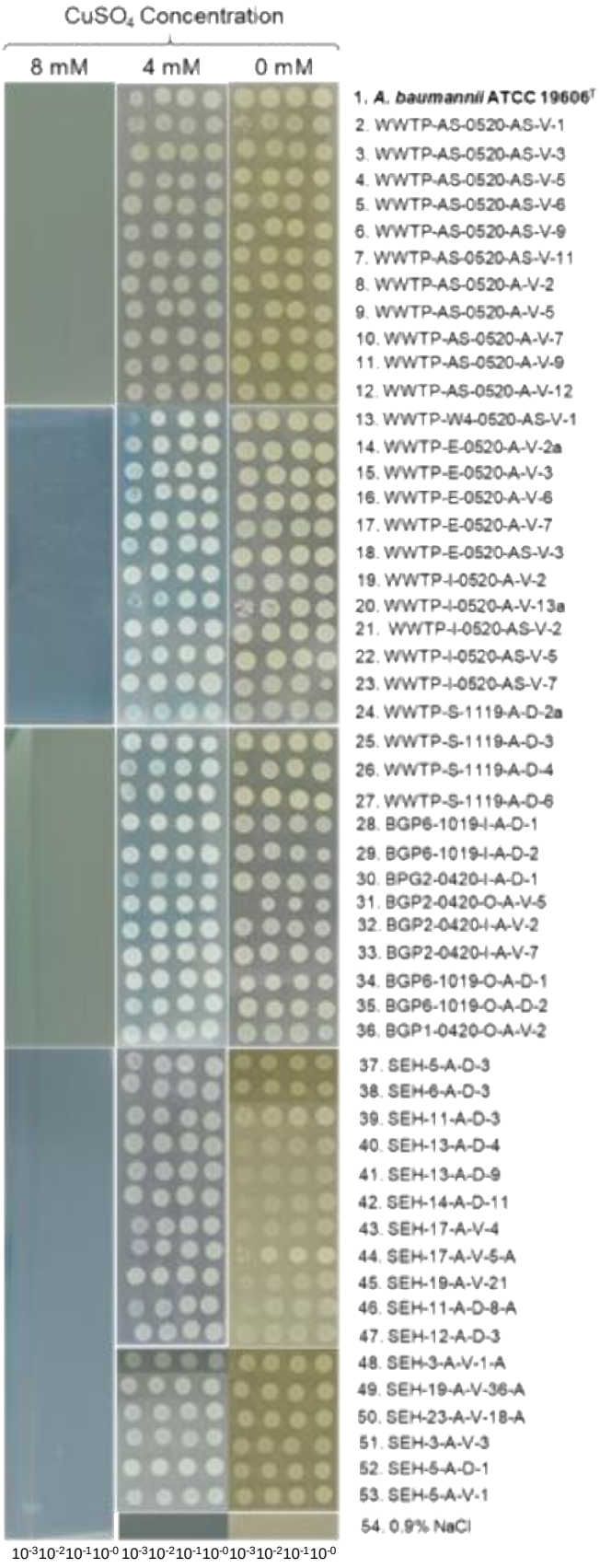


Figure S6. Copper tolerance of *A. baumannii* strains tested by pipetting 5 µL of serial dilution (10^0 – 10^{-3} dilution, turbidity adjusted to standard 0.5 McFarland) of overnight

cultured isolates on Mueller Hinton agar plates supplemented with 4, 8, 12, 16, 20, 24, 32 and 36 mM $CuSO_4$ $5H_2O$ adjusted to pH 7.2 (MERCK, Germany). The plates were incubated at under oxic conditions at 37°C and checked for growth after 24h of incubation. The lowest copper concentration that suppressed growth was considered as tMIC value. The growth was completely suppressed in concentration of 8 mM and above (data not shown).

Supplementary references

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Annex

Chapter III, Supporting Information

Acinetobacter baumannii in manure and anaerobic digestates of German biogas plants

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Supplemtary tables

Table S1. Characteristics of the studied German BGPs. All plants are continuous stirred tank reactors (CSTRs). Composition of input material and digestion system of the studied biogas plants (BGPs) adpated from Schauss et al. 2016.

	BGP-1	BGP-2	BGP-3	BGP-4	BGP-5	BGP-6	BGP-7	BGP-8	BGP-9	BGP-10	BGP-11	BGP-12	BGP-13	BGP-14	BGP-15
Plant type															
Wet Fermenter	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Temperature															
mesophile	x	x	x	x	x	x	x	x	x	x	x		x	x	x
thermophile												x			
Process stages															
One-stage					x	x		x	x						
Two-stage	x	x	x	x			x			x	x		x	x	x
Multi-stages												2 lines			
Volume (m^3)															
Reception tank	250	150	110	200	120	100	200	26		400	300	2 x 500	150	150	100
Fermenter	1800	1500	1500	1700	1700	2000	1200	1400	600	2000	2x 1400	2 x 4000	1100	1200	500
Post-Fermenter	3300	2500	2700	2200			1200	0		2000	1000	2 x 4000	2700	2200	500
Storage tank	-	5000	1000	1000	3200	4000	3000	2488	2450	6400	2x 3150	2 x 5000			1500
Agitation technology															
Mechanical, slow, central		x	x								x			x	
Mechanical, fast, decentralized					x	x		x	x	x		x	x		x

Mechanical, paddle-agitator, longitudinal hydraulic	2		2	2			2			x			x x	x	
Retention time [days]															
Reception tank	120	7		7			5	24	42	30		0.5			
Fermenter	80		50	50	80	60-70	35	110		50	50	36	75	90-100	~30
Post-Fermenter	180	110	90	50			35			50	17	36	160	70	30
Storage tank				120	90	180		180	240		108	46			70
Total	**380**	**110**	**140**	**227**	**170**	**240**	**75**	**270**		**100**	**175**	**118**		**170**	**130**

Table S2. Realtime PCR based absolute quantification of *Acinetobacter* and *Bacteria* 16S rRNA gene copies in input and output samples of the studied German biogas plants. Given values represent mean values and standard deviations of three biological replicates (independent total community DNA extracts).

		16S rRNA gene copies (g×FW)$^{-1}$ Mean(±SD)		**Normalisation**
Biogas plant	Sampling time	*Acinetobacter*	*Bacteria*	*Acinetobacteria*/ *Bacteria* 16S rRNA gene copies
BGP-1 (I)	03.07.2012	$1.9(\pm0.9)\times10^{8}$	$3.2(\pm0.9)\times10^{10}$	6.43×10^{-3}
BGP-2 (I)	02.07.2012	$2.1(\pm0.1)\times10^{8}$	$3.4(\pm0.8)\times10^{10}$	6.03×10^{-3}
BGP-3 (I)	06.03.2012	$1.7(\pm0.0)\times10^{6}$	$7.2(\pm9.9)\times10^{10}$	7.05×10^{-5}
BGP-4 (I)	05.03.2012	$2.0(\pm0.8)\times10^{8}$	$5.0(\pm1.1)\times10^{10}$	4.23×10^{-3}
BGP-5 (I)	04.07.2012	$1.8(\pm1.0)\times10^{8}$	$3.3\pm0.6)\times10^{10}$	5.09×10^{-3}
BGP-6 (I)	04.09.2012	$1.0(\pm0.5)\times10^{8}$	$2.8(\pm0.9)\times10^{10}$	3.88×10^{-3}
BGP-7 (I)	07.03.2012	$3.1(\pm3.0)\times10^{7}$	$4.3(\pm1.1)\times10^{10}$	6.60×10^{-4}
BGP-8 (I)	07.05.2012	$1.0(\pm0.3)\times10^{8}$	$2.5(\pm0.4)\times10^{10}$	4.29×10^{-3}
BGP-9 (I)	08.05.2012	$1.2(\pm0.6)\times10^{8}$	$3.1(\pm1.0)\times10^{10}$	4.02×10^{-3}
BGP-10 (I)	09.05.2012	$1.3(\pm0.6)\times10^{8}$	$7.4(\pm2.7)\times10^{10}$	2.24×10^{-3}
BGP-11 (I)	09.05.2012	$7.8(\pm7.4)\times10^{7}$	$2.3(\pm0.9)\times10^{10}$	3.00×10^{-3}
BGP-12 (I)	05.09.2012	$8.9(\pm7.1)\times10^{6}$	$2.6(\pm1.5)\times10^{10}$	4.19×10^{-4}
BGP-13 (I)	08.10.2012	$4.9(\pm4.1)\times10^{7}$	$1.6(\pm1.3)\times10^{10}$	2.48×10^{-3}
BGP-14 (I)	09.10.2012	$9.4(\pm6.8)\times10^{7}$	$2.3(\pm1.2)\times10^{10}$	4.04×10^{-3}
BGP-15 (I)	10.10.2012	$9.7(\pm8.5)\times10^{7}$	$2.8(\pm0.7)\times10^{10}$	3.86×10^{-3}
BGP-1 (O)	03.07.2012	$9.5(\pm2.7)\times10^{5}$	$2.6(\pm1.6)\times10^{10}$	3.09×10^{-5}
BGP-2 (O)	02.07.2012	$1.3(\pm0.5)\times10^{6}$	$5.4(\pm3.6)\times10^{10}$	3.39×10^{-5}
BGP-3 (O)	06.03.2012	$2.4(\pm0.7)\times10^{5}$	$1.7(\pm0.5)\times10^{10}$	1.42×10^{-5}
BGP-4 (O)	05.03.2012	$6.3(\pm3.2)\times10^{5}$	$2.6(\pm0.7)\times10^{10}$	2.72×10^{-5}
BGP-5 (O)	04.07.2012	$6.0(\pm3.0)\times10^{5}$	$2.0(\pm0.4)\times10^{10}$	2.99×10^{-5}
BGP-6 (O)	04.09.2012	$1.7(\pm0.9)\times10^{6}$	$2.6(\pm0.4)\times10^{10}$	7.15×10^{-5}
BGP-7 (O)	07.03.2012	$1.6(\pm1.5)\times10^{5}$	$1.1(\pm0.1)\times10^{10}$	1.42×10^{-5}
BGP-8 (O)	07.05.2012	$1.6(\pm0.4)\times10^{6}$	$3.2(\pm1.1)\times10^{10}$	5.49×10^{-5}
BGP-9 (O)	08.05.2012	$2.9(\pm1.3)\times10^{6}$	$2.3(\pm1.0)\times10^{10}$	1.41×10^{-4}
BGP-10 (O)	09.05.2012	$1.1(\pm0.5)\times10^{6}$	$7.7(\pm2.3)\times10^{10}$	1.40×10^{-5}
BGP-11 (O)	09.05.2012	$1.1(\pm0.5)\times10^{6}$	$3.0(\pm0.6)\times10^{10}$	3.53×10^{-5}
BGP-12 (O)	05.09.2012	$1.0(\pm1.0)\times10^{6}$	$3.2(\pm0.9)\times10^{10}$	3.50×10^{-5}
BGP-13 (O)	08.10.2012	$1.2(\pm0.7)\times10^{6}$	$3.5(\pm0.5)\times10^{10}$	3.25×10^{-5}
BGP-14 (O)	09.10.2012	$8.6(\pm7.7)\times10^{5}$	$1.3(\pm0.5)\times10^{10}$	5.95×10^{-5}
BGP-15 (O)	10.10.2012	$1.4(\pm0.1)\times10^{6}$	$2.9(\pm0.6)\times10^{10}$	4.81×10^{-5}

BGP, Biogas plant; I, Input; O, Output; FW, fresh weight in grams

Table S3. Minimum inhibitory concentrations (MICs) of 12 different antibiotics for *Acinetobacter* spp. isolates following test carried in Micronaut-S plates (Merlin, Bornheim-Hersel) using the CLSI guidelines M100-S23 (CLSI 2013) after 24h of incubation. For each antibiotic a concentration range of eight concentrations was tested [values in mg L-1]; for: amoxicillin (0.5-64), cefquinome (0.25-32) (+/- clavulanic acid), ceftiofur (0.25-32) (+/- clavulanic acid), enrofloxacin (0.0625-8), florfenicol (0.5-64), oxacillin (0.25-32), sulfamethoxazole (4-256), tetracycline (0.25-32), trimethoprim/sulfamethoxazole (0.0625/1.125-8/125) and tylosin (0.125-16).

	Isolates	**Phylogenetic assignment (16S rRNA gene similarity)**	**AMX**	**OXA**	**CET**	**CUC**	**CEQ**	**CQC**	**ENR**	**TET**	**TLS**	**FLL**	**T/S**	**SMO**
1	**KPC-SM-21**	97%, *A.baumannii* ATCC 19606^T	S	>32	8	8/4	8	4/4	0.5	S	>16	16	S	<4
2	551B1-12EESBL	100%, *A.calcoaceticus* DSM 30006^T	S	>32	16	8/4	2	2/4	0.125	S	>16	64	S	<4
3	**552B1-12EESBL**	99.9%, *A.baumannii* ATCC 19606^T	S	>32	16	16/4	2	2/4	<0.0625	S	>16	>64	S	<4
4	553B1-12EESBL	99.9%, *A.calcoaceticus* DSM 30006^T	S	>32	16	16/4	4	4/4	0.125	S	>16	64	S	<4
5	**571B5-12EESBL**	99.8%, *A.baumannii* ATCC 19606^T	S	>32	32	32/4	4	8/4	0.125	S	>16	>64	S	<4
6	574B5-12EESBL	98.2%, *A.guillouiae* CIP 63.46^T	S	>32	4	1/4	1	<0.25/4	<0.0625	S	>16	2	S	<4
7	734B5-12EEMB	99.6%, *A.beijerinckii* CIP 110307^T	S	16	2	<0.25/4	<0.25	<0.25/4	<0.0625	S	>16	1	S	>256
8	794B1-12ER2A	99.5%, *A.indicus* CIP 110367^T	S	4	1	<0.25/4	<0.25	<0.25/4	<0.0625	S	>16	2	S	8
9	**901B6-12EESBL**	99.7%, *A.baumannii*	S	>32	16	16/4	4	4/4	0.125	S	>16	>64	S	>256

		ATCC 19606^{T}												
10	902B6-12EESBL	99.5%, *A.bereziniae* LMG 1003^{T}	S	>32	2	2/4	0.5	1/4	0.125	S	>16	8	S	<4
11	**945B12-12AESBL**	99.7%, *A.baumannii* ATCC 19606^{T}	S	>32	16	16/4	4	4/4	0.125	S	>16	>64	S	32
12	815B5-12E-R2A	99.3%, *A.towneri* DSM 14962^{T}	S	32	2	0.5/4	<0.25	<0.25/4	<0.0625	S	>16	1	S	<4

ß-Lactam antibiotics: AMX= amoxicillin, OXA= oxacillin, CET= ceftiofur, CUC= ceftifour+clavulanic acid, CEQ= cefquinom, CQC= cefquinom+clavulanic acid; fluoroquinolones: ENR= enrofloxacin; polyketide broad spectrum: TET= tetracyclines; macrolides: TLS= tylosin; thiamphenicol analogue: FLL= florfenicol; DHFR inhibitor: T/S= trimethoprim/SMO; Sulfonamide: SMO= sulfamethoxazole. Strains of A. baumannii are highlighted in bold. S, susceptible phenotype to specific antibiotic according to EUCAST and CLSI clinical breakpoints. MIC values are given for antibiotics lacking clinical breakpoints

Table S4. Minimum inhibitory concentrations (MICs) of all six strains of *A. baumannii* was tested in Micronaut-S MDR MRGN-Screening 3 system (Merlin, Bornheim-Hersel) using using the CLSI guidelines M100-S23 (Clinical and Laboratory Stabdards Institute 2013). S, susceptible phenotype to specific antibiotic according to EUCAST and CLSI clinical breakpoints. MIC values (mg L-1 concentration of antibiotics) are given for antibiotics lacking clinical breakpoints.

	Isolates/Strains	LEV	TGC	CIP	COL	FOS	CMP	T/S	TMO	CTX	AMK	CAZ	CZB	CTA	IMP	CAA	MER	MEE	MEB	PIT	PIP
1	KPC-SM-17a	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	<1/4	S	<0,25	0,5	S	S
2	KPC-SM-125	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	2/4	S	<0,25	4	S	S
3	552B1-12EESBL	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	<1/4	S	<0,25	1	S	S
4	571B5-12EESBL	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	2/4	S	<0,25	1	S	S
5	901B6-12EESBL	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	<1/4	S	<0,25	1	S	S
6	945B12-12AESBL	S	<0.25	S	S	>64	>16	S	128	S	S	S	S	<1/4	S	<1/4	S	<0,25	1	S	S

Antibiotics: TMO=temocillin, PIP=piperacillin, PIT=piperacillin/tazobactam, CTX=cefotaxime, CAZ=ceftazidime, CAA=ceftazidime/avibactam, CZB=ceftazidime/3-APB, CTA=ceftolozan/tazobactam, IMP=imipenem, MER=meropenem, MEE=meropenem/EDTA, MEB=meropenem/3-APB, AMK=amikacin, TGC=tigecycline, CMP=chloramphenicol, FOS=fosfomycin, T/S=trimethoprim/sulfamethoxazol, CIP=ciprofloxacin, LEV=levofloxacin and COL=colistin. APB: aminophenylboronic acid.

Table S5. Overview of chromosomally encoded multidrug efflux system and beta-lactamase genes of *A. baumannii* strains. Comparative genomic analysis was performed in EDGAR 2.3 (Blom et al. 2016) using the genomes of *A. baumannii* ATCC 19606^T (as reference) and six *A. baumannii* strains from this study RND: resistance-nodulation-cell division familiy; MATE: multidrug and toxic compound extrusion family.

					A. baumannii strain						
Gene	Protein	Family	function	Reference for evidence	ATCC 19606 T	552B12-12EESBL	KPC-SM-17a	KPC-SM-125	945B12-12AESBL	901B6-12EESBL	571B5-12EESBL
					Locus tag number						
adeRS	Two-component system AdeRS	RND efflux	regulation of expression of AdeABC efflux pump, virulence and biofilm	Marchand *et al*. 2004; Yoon, Courvalin and Grillot-Courvalin. 2013; Richmond *et al.* 2016	HMPREF0010_00591-00592	AB552B1_02502-02503	ABKPCSM17A_03207-03208	ABKPCSM125_02328-02329	AB945B12_00355-00354	AB901B6_02079-02080	AB571B5_02451-02450
adeA	AdeA, component of tripartite efflux pump AdeABC		resistance to aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, and trimethoprim; reduced susceptibility to tigecycline	Yoon *et al*. 2013; Magnet, Courvalin and Lambert 2001; Peleg, Adams and Paterson. 2007; Ruzin, Keeney and Bradford. 2007; Ruzin, Immermann and Bradford 2010	HMPREF0010_00593	AB552B1_02504	ABKPCSM17A_03209	ABKPCSM125_02330	AB945B12_00356	AB901B6_02078	AB571B5_02452
adeB	AdeB, component of tripartite efflux pump AdeABC				HMPREF0010_00594	AB552B1_02505	ABKPCSM17A_03210	ABKPCSM125_02331	AB945B12_00357	AB901B6_02077	AB571B5_02453
adeC	AdeC, component of tripartite efflux pump AdeABC				Absent	Absent	Absent	ABKPCSM125_02332	Absent	Absent	Absent
abaF	AbaF, major facilitator superfamily transporter (MFS)		fosfomycin resistance efflux	Sharma *et al.* 2017	HMPREF0010_01090	AB552B1_01813	ABKPCSM17A_02952	ABKPCSM125_02646	AB945B12_02844	AB901B6_00760	AB571B5_01365

ampC	Ampc-type cephalosporinase	class C beta lactamase	beta lactam resistance	Corvec *et al*. 2003	HMPREF0010_02318	AB552B1_02062	ABKPCSM17A_00981	ABKPCSM125_01623	AB945B12_00660	AB901B6_03235	AB571B5_03198
craA	chloramphenicol resistance protein CraA	Non-RND efflux	chloramphenicol resistance	Roca *et al*. 2009	HMPREF0010_02562	AB552B1_01518	ABKPCSM17A_01321	ABKPCSM125_03508	AB945B12_01917	AB901B6_01337	AB571B5_00725
abeM	AbeM, mutidrug efllux	MATE efflux	norfloxacin, ofloxacin, ciprofloxacin, and gentamicin resistance	Su *et al*. 2005; Vila, Martí and Sánchez-Céspedes. 2007	HMPREF0010_01847	AB552B1_01285	ABKPCSM17A_01543	ABKPCSM125_00210	AB945B12_01545	AB901B6_01787	AB571B5_00201
adeIJK	AdeIJK	RND efflux	resistance to beta lactams (for e.g., ticarcillin, cephalosporins, and aztreonam), fluoroquinolones, tetracyclines, tigecycline and chloramphenicol	Damier-Piolle *et al*. 2008	HMPREF0010_02880-02882	AB552B1_03110-03112	ABKPCSM17A_02658-02660	ABKPCSM125_00755-00757	AB945B12_02298-02300	AB901B6_02914-02916	AB571B5_01795-01797

Table S6. Overview of chromosomally encoded virulence factors of *A. baumannii* strains. Comparative genomic analysis was performed in EDGAR 2.3 (Blom et al. 2016) using the genomes of *A. baumannii* ATCC 19606^T and six *A. baumannii* strains from this study. Several virulence factors were identified in *A. baumannii* strains of this study, and major virulence factors required to thrive in both host and abiotic environment are listed in the table below.

					A. baumannii						
Gene/Locus/ Operon	Protein	Predicted function	Role in pathogenesis	Reference for evidence	ATCC 19606T	552B12-12EESBL	KPC-SM-17a	KPC-SM-125	945B12-12AESBL	901B6-12EESBL	571B5-12EESBL
Type II secretion system					Locus tag						
gspN	T2SS protein GspN	Unknown function	In vivo survival	Johnson *et al*. 2016 Elhosseiny *et al*. 2016 Harding *et al*. 2016; Harding, Hennon and Feldman. 2018	HMPREF0010_01960	AB552B1_01163	ABKPCSM17A_01147	ABKPCSM125_00332	AB945B12_01423	AB901B6_01910	AB571B5_00080
gspC	T2SS protein GspC	Inner-membrane platform protein			HMPREF0010_01959	AB552B101164	ABKPCSM17A_01148	ABKPCSM125_00331	AB945B12_01424	AB901B601909	AB571B500081
gspD	T2SS protein GspD	Outer-membrane secretion			HMPREF0010_01958	AB552B1_01165	ABKPCSM17A_01149	ABKPCSM125_00330	AB945B12_01425	AB901B601908	AB571B500082
gspL	T2SS protein GspL	Inner-membrane platform protein			HMPREF0010_00073	AB552B1_02167	ABKPCSM17A_02152	ABKPCSM125_01736	AB945B12_00544	AB901B603350	AB571B503088
gspM	T2SS protein GspM	Inner-membrane platform protein			HMPREF0010_00074	AB552B1_02168	ABKPCSM17A_02151	ABKPCSM125_01737	AB945B12_00543	AB901B603351	AB571B503087
gspE	T2SS protein GspE	Secretion ATPase			HMPREF0010_01637	AB552B102134	ABKPCSM17A_02185	ABKPCSM125_01703	AB945B12_00582	AB901B603312	AB571B503121
gspG	T2SS protein GspG	Major pseudopilin			HMPREF0010_01869	AB552B1_01262	ABKPCSM17A_01520	ABKPCSM125_00233	AB945B12_01522	AB901B6_01810	AB571B5_00178
gspF	T2SS protein GspF	Inner-membrane platform protein			HMPREF0010_01870	AB552B1_01261	ABKPCSM17A_01519	ABKPCSM125_00234	AB945B12_01521	AB901B6_01811	AB571B5_00177
gspKJIH	T2SS protein GspKJIH	Minor pseudopilins			HMPREF0010_00793-00796	AB552B1_02731-02734	ABKPCSM17A_00529-00532	ABKPCSM125_02540-02543	AB945B12_03518-03521	AB901B6_00498-00501	AB571B5_02659-02662
Csu system extrudes a type I chaperone-–usher pilus as virulence factor					Locus tag						
csuA/BABCDE locus	Csu pili	extrudes a type I chaperone-usher pilus	formation and maintenance of biofilms, adherence	Tomaras *et al*. 2003; 2008	HMPREF0010_00109-00114	AB571B5_03029-03034	ABKPCSM17A_03786-03791	ABKPCSM125_01788-01793	AB945B12_00026-00031	***Absent in Genome***	AB571B5_03029-03034
Type 6 secrertion system as virulence factor					Locus tag						
tssL	T6SS protein TssL	membrane complex proteins		Weber *et al*. 2013 Carruthers *et al*. 2013	HMPREF0010_01111	AB552B1_01835	ABKPCSM17A_02975	ABKPCSM125_02624	AB945B12_02866	AB901B6_00782	AB571B5_01387

tssK	T6SS protein TssK	baseplate components	interspecies competition, host colonisation	Wright *et al*. 2014 Jones *et al.* 2015 Repizo *et al.* 2015	HMPREF0010_01112	AB552B1_01836	ABKPCSM17A_02976	ABKPCSM125_02623	AB945B12_02867	AB901B6_00783	AB571B5_01388
tssA	T6SS protein TssA	Priming protein			HMPREF0010_01113	AB552B1_01837	ABKPCSM17A_02977	ABKPCSM125_02622	AB945B12_02868	AB901B6_00784	AB571B5_01389
tssH	T6SS protein TssH	ATPase ClpV			HMPREF0010_01114	AB552B1_01838	ABKPCSM17A_02978	ABKPCSM125_02621	AB945B12_02869	AB901B6_00785	AB571B5_01390
PAAR domain coding gene	proline-alanine-alanine arginine repeat superfamily	Structural and effector function			HMPREF0010_01115	AB552B1_01840	ABKPCSM17A_02979	ABKPCSM125_02620	AB945B12_02870	AB901B6_00786	AB571B5_01391
tagF	T6SS protein TagF	Posttranslational repressor of T6SS			HMPREF0010_01117	AB552B1_01842	ABKPCSM17A_02981	ABKPCSM125_02618	AB945B12_02872	AB901B6_00788	AB571B5_01393
tssM	T6SS protein TssM	membrane complex proteins			HMPREF0010_01118	AB552B1_01843	ABKPCSM17A_02982	ABKPCSM125_02617	AB945B12_02873	AB901B6_00789	AB571B5_01394
tssG	T6SS protein TssG	baseplate components			HMPREF0010_01120	AB552B1_01845	ABKPCSM17A_02984	ABKPCSM125_02615	AB945B12_02875	AB901B6_00791	AB571B5_01396
tssF	T6SS protein TssF	baseplate components			HMPREF0010_01121	AB552B1_01846	ABKPCSM17A_02985	ABKPCSM125_02614	AB945B12_02876	AB901B6_00792	AB571B5_01397
tssE	T6SS protein TssE	baseplate components			HMPREF0010_01122	AB552B1_01847	ABKPCSM17A_02986	ABKPCSM125_02613	AB945B12_02877	AB901B6_00793	AB571B5_01398
hcp	Hemolysin coregulated protein Hcp	tubule protein			HMPREF0010_01123	AB552B1_01848	ABKPCSM17A_02987	ABKPCSM125_02612	AB945B12_02878	AB901B6_00794	AB571B5_01399
tssB	T6SS protein TssB	Sheath components			HMPREF0010_01125	AB552B1_01850	ABKPCSM17A_02989	ABKPCSM125_02610	AB945B12_02880	AB901B6_00796	AB571B5_01401
tssC	T6SS protein TssC	Sheath components			HMPREF0010_01124	AB552B1_01849	ABKPCSM17A_02988	ABKPCSM125_02611	AB945B12_02879	AB901B6_00795	AB571B5_01400
vgrG	Valine-glycine repeat protein G	structural and effector function			HMPREF0010_03005	AB552B1_01441	ABKPCSM17A_00951	ABKPCSM125_01237	AB945B12_03122	AB901B6_00912	AB571B5_01065
Capsule, biofilm and metal acquisition systems as virulence factor					Locus tag						
OmpA (Omp38)	Outer membrane protein A	Outer membrane protein	Adherence, invasion, apoptosis, biofilm, persistence	Choi *et al*. 2005, 2008a, 2008b; Gaddy, Tomaras and Actis 2009; Lee *et al*. 2010; Smani *et al*. 2014; Wang *et al.* 2014	HMPREF0010_02782	AB552B1_03226	ABKPCSM17A_02419	ABKPCSM125_00647	AB945B12_00825	AB901B6_02799	AB571B5_01903
ptK	protein tyrosine kinase	Capsular polymerisation and assembly	Biofilm, tissue infection, serum growth	Russo *et al.* 2010	HMPREF0010_03290	AB552B1_03541	ABKPCSM17A_00094	ABKPCSM125_03000	AB945B12_03257	AB901B6_03546	AB571B5_03567
epsA	putative polysaccharide export outer membrane protein	Capsular polymerisation and assembly	Human serum resistance, in vivo survival	Russo *et al*. 2010	HMPREF0010_03288	AB552B1_03539	ABKPCSM17A_00096	ABKPCSM125_02998	AB945B12_03259	AB901B6_03548	AB571B5_03569
nfuA	Fe/S protein NfuA	iron-sulfur cluster biogenesis in iron deficiency	survival in vivo, hosts cell attack, persistence	Zimbler *et al*. 2012	HMPREF0010_01516	AB552B1_00389	ABKPCSM17A_03396	ABKPCSM125_00929	AB945B12_01036	AB901B6_01068	AB571B5_01641

Table S7. ST-types of the six strains of *A. baumannii* based on Pasteur and Oxford MLST schemes. Cpn60: Chapheronin 60 KDa; FusA: Elongation factor G; GltA: Citrate synthase; PyrG: CTP synthase; RecA: homologous recombination factor A; RplB: 50S ribosomal protein L2; RpoB: RNA polymerase β-subunit; GyrB: DNA gyrase subunit B; GdhB: glucose dehydrogenase B; Gpi: glucose-6-phosphate isomerase and RpoD: RNA polymerase sigma 70 factor. Novel STs and alleles are listed in bold font.

		Pasteur scheme (Diancourt *et al*. 2010)							**Oxford scheme** (Bartual *et al*. 2005)							
Isolates	Source	ST-types	Housekeeping gene allele number						ST-types	Housekeeping gene allele number						
			cpn60	*fusA*	*gltA*	*pyrG*	*recA*	*rplB*	*rpoB*		*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*
KPC-SM-17a	BGP-001 (I)	**ST-1442**	8	1	2	1	9	1	4	**Novel**	1	52	4	11	32	205	6
KPC-SM-125	BGP-015 (O)	**ST-1443**	1	49	7	3	12	1	5	ST1210^{Ox}	21	12	68	41	4	103	4
552B1-12EESBL	BGP-001 (I)	**ST-1444**	3	2	148	1	137	1	5	**Novel**	106	93	181	60	1	**Novel**	130
571B5-12EESBL	BGP-005 (I)	ST46^{P}	5	12	11	2	14	9	14	ST1557^{Ox}	31	33	67	40	16	142	7
901B6-12EESBL	BGP-006 (I)	**ST-1445**	3	3	17	1	137	1	4	**Novel**	60	**Novell**	**Novel**	12	1	38	**Novel**
945B12-12AESBL	BGP-012 (O)	**ST-1446**	1	2	11	54	9	1	2	**Novel**	31	98	**Novel**	11	4	66	76

Supplementary figures

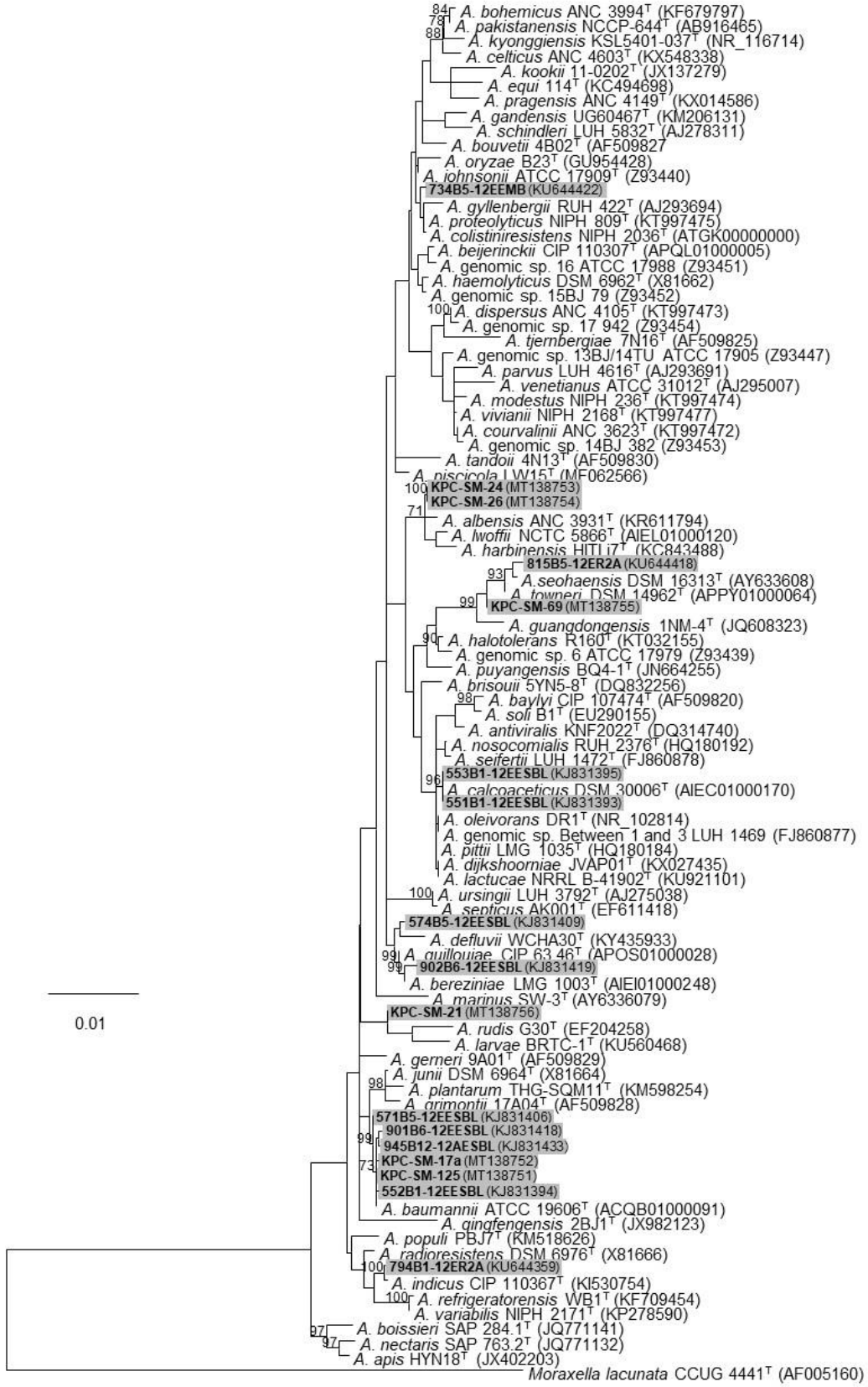

Figure S1. Phylogenetic placement of different isolates within genus *Acinetobacter,* based on full-length 16S rRNA gene sequence. The evolutionary history was inferred

using the Maximum-Likelihood method (Felsenstein, 1981) based on the Kimura 2-parameter model (Kimura, 1980) using discrete Gamma distribution (+G) model with 5 categories to model evolutionary rates among the sites and assuming certain fraction of sites were evolutionary invariables (+I) respectively. Bootstrap values >70 % (100 resamplings) were indicated. *Moraxella lacunata* CCUG 4441^{T} (AF005160) was used as an outgroup. Bar, 0.01 nt substitutions per sequence position. *Acinetobacter* sp. isolates from biogas plants were represented in bold font. GenBank accession numbers were given in parentheses.

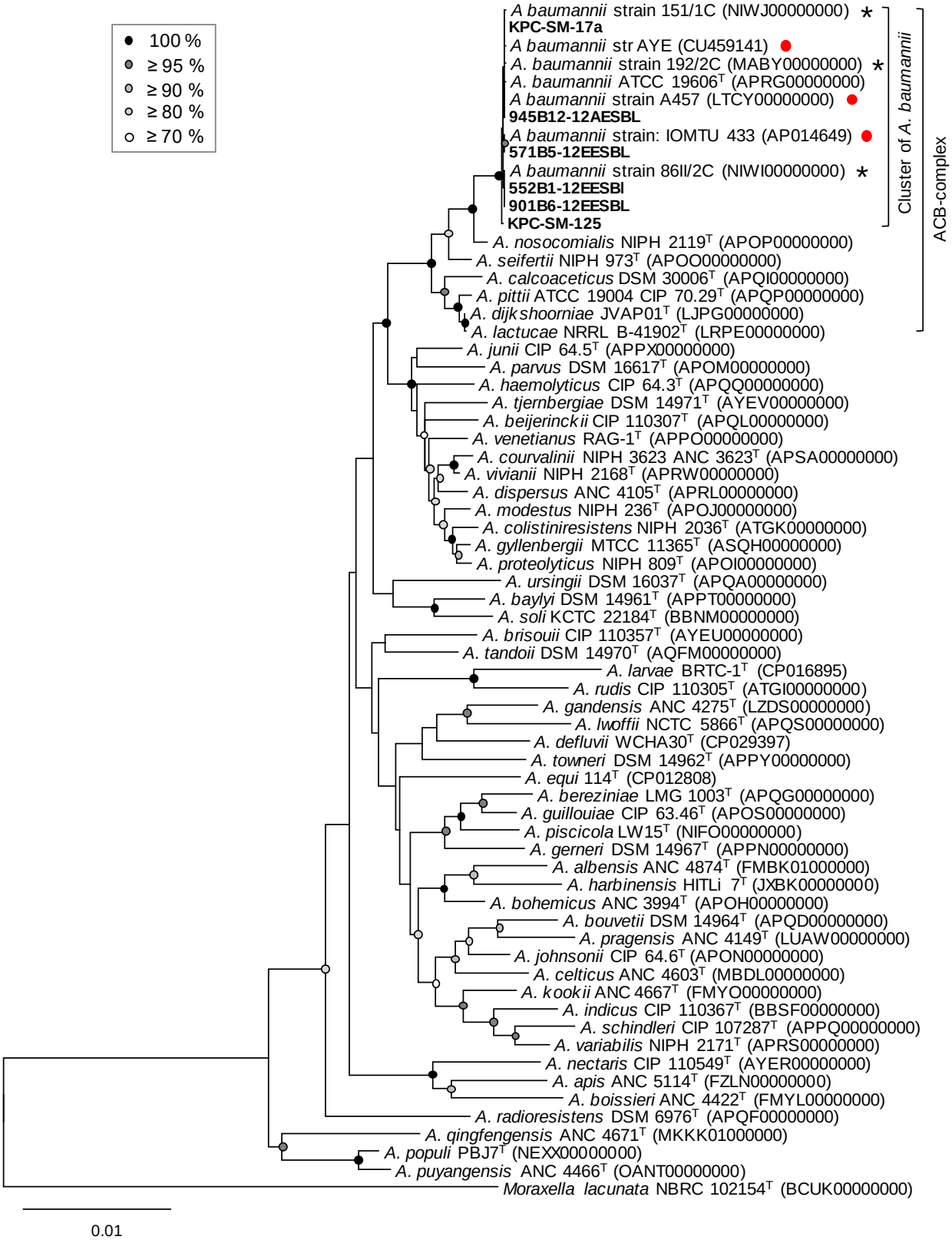

Figure S**2**. Maximum likelihood tree based on cocatenated nucleotide sequences of protein coding genes (MLSA analysis) of the Pasteur MLST scheme (Diancourt *et al*. 2010). The General Time Reversible Model (Nei and Kumar. 2000). *A. baumannii*

strains of biogas plants, avian sample (Wilharm *et al*. 2017) and clinical source, were included in the analysis. MLSA is based upon concatenated genes listed in MNode represents ≥70% (100 replications) bootstrap values. Type strain of *Moraxella lacunata* is used as outgroup. Asterisks and red dots indicate avian and clinical strains. Bar represents 0.01 sequence divergence. Strains from this study are represented in bold font.

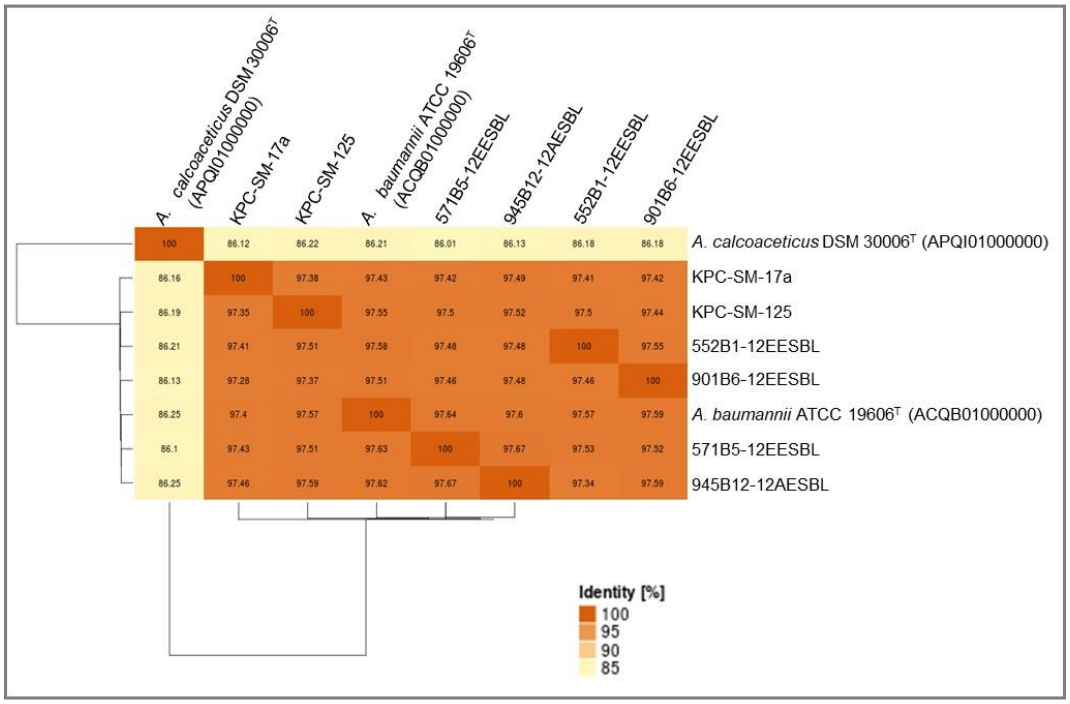

Figure S3. Cluster analyses based on pairwise ANI values determined for all *A. baumannii* strains of this study and respective type strains of *A. baumannii*. Values are given in percentages. Genome of *A. calcoaceticus* DSM 30006^T was used to root the cluster. The genome accession numbers of all *A. baumannii* strains are uploaded in NCBI under bioproject PRJEB35515.

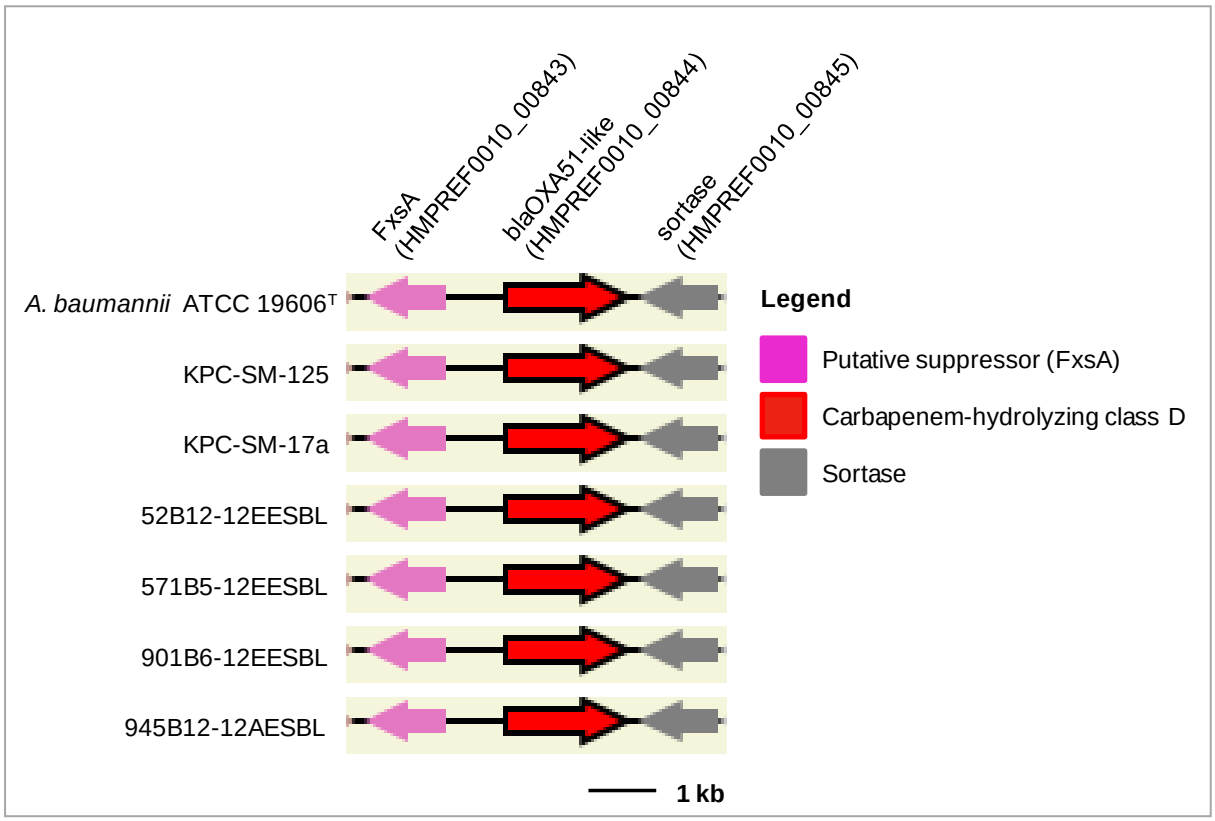

Figure S4. Distribution of *bla*$_{\text{OXA-51-like}}$ gene in genome of *A. baumannii* strains. The complete genome of *A. baumannii* ATCC 19606^{T} is used as reference. Lack of insertion of IS*Aba1* (insertion sequence) in upstream of OXA-51 genes. Locus tag of specific gene is given in parenthesis.

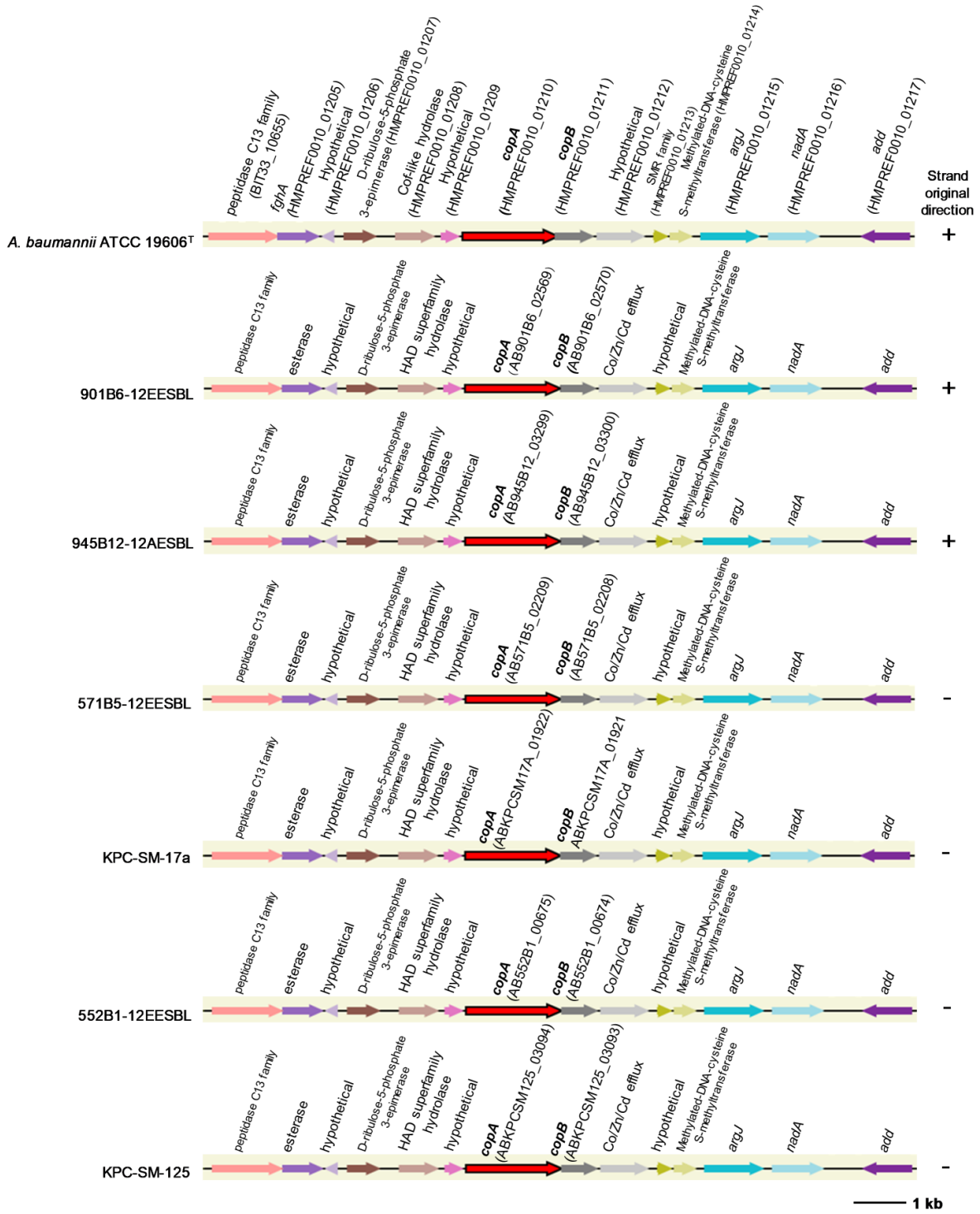

Figure S5. Distribution of copper related efflux loci in the genome of all six strains of *A. baumanniii* isolated from German biogas plants. The total DNA of *A. baumannii* ATCC 19606^{T} was used as reference genome. Copper efflux genes were highlighted in bold. Locus tag numbers were given in parentheses.

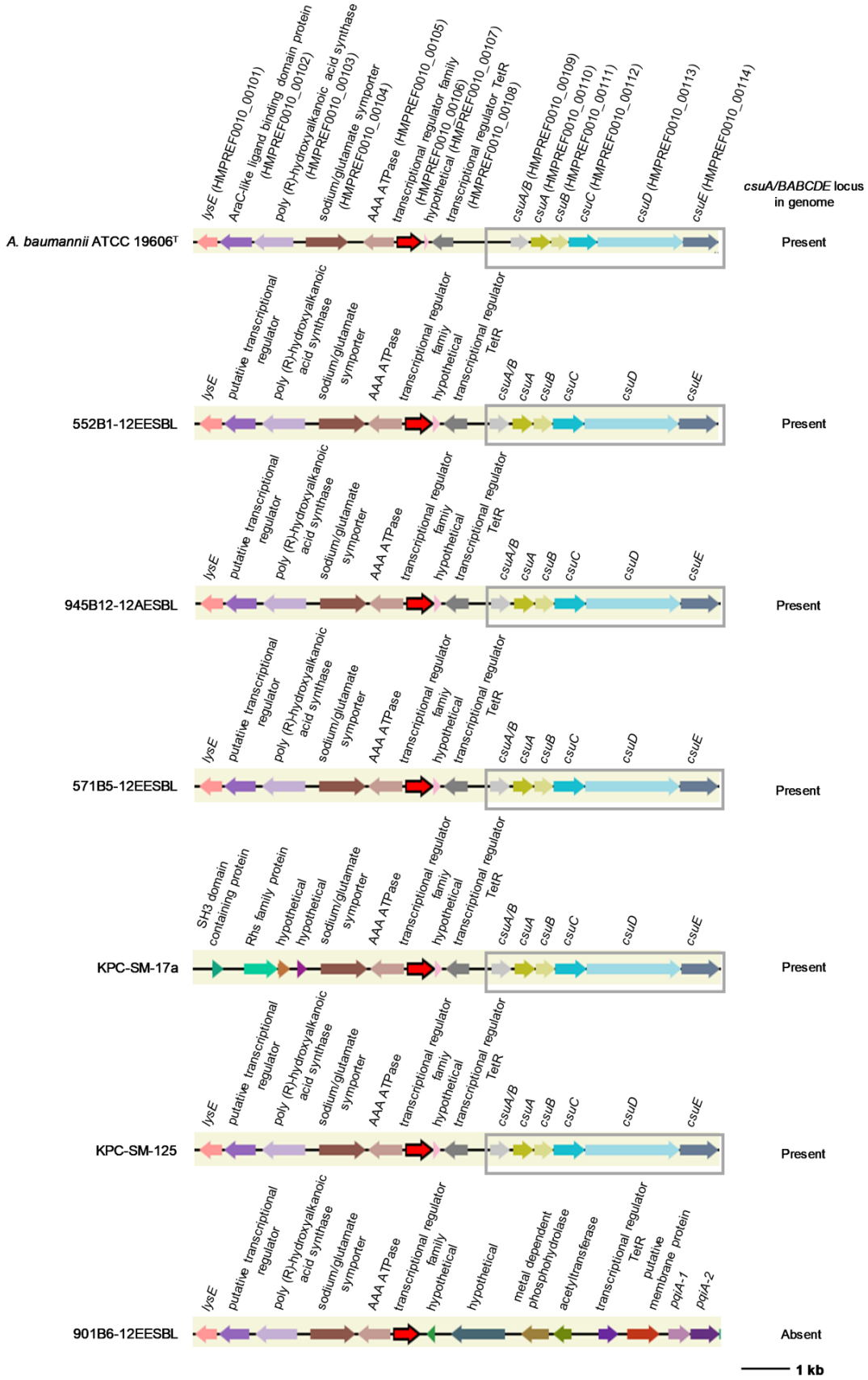

Figure S6 Distribution of type I chaperone-usher pilus (Csu pili) system locus in the genome of *A. baumanniii* strains isolated from German biogas plants. The genome of

901B6-12EESBL lacked the Csu pili. The genome of *A. baumannii* ATCC 19606^{T} was used as reference genome. Locus tag numbers were given in parentheses. Csu pili system (*csuA/BABCDE* locus) was shown inside of rectangular box.

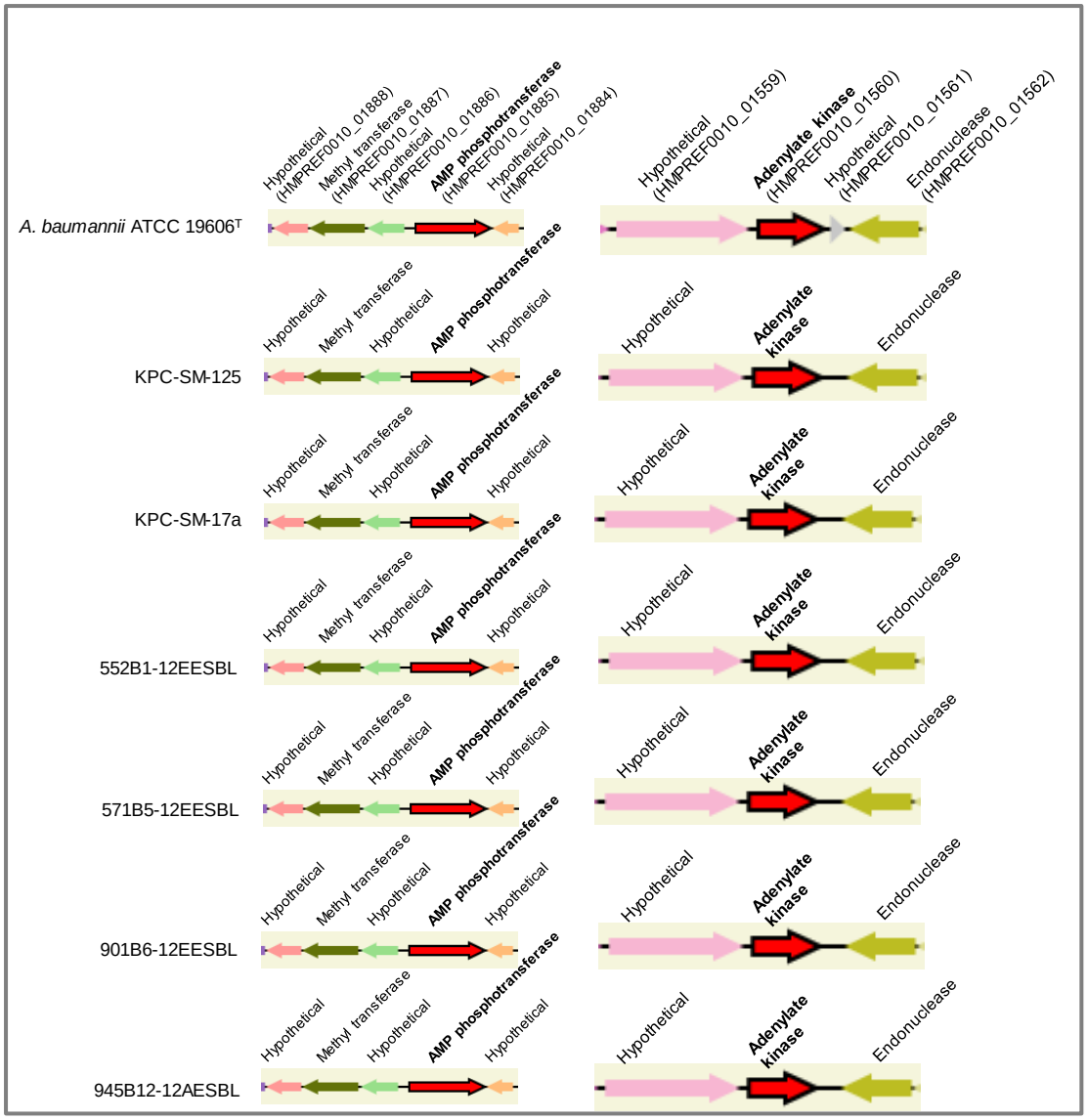

Figure S7. Distribution of genetic loci of AMP phosphotransferase and adenylate kinase in the genome of *A. baumanniii* strains isolated from German biogas plants. The genome of *A. baumannii* ATCC 19606^{T} is used as reference genome. Locus tag of specific gene is given in parenthesis. Locus tag numbers of AMP phosphotransferase for KPC-SM-125, KPC-SM-17a, 552B1-12EESBL, 571B5-12EESBL, 901B6-12EESBL, and 945B12-12AESBL are as follows: ABKPCSM125_00249, ABKPCSM17A_01503, AB552B1_01246, AB571B5_00162, AB901B6_01826, and AB945B12_01506. Locus tag numbers of adenylate kinase are ABKPCSM125_00974, ABKPCSM17A_03352, AB552B1_00346, AB571B5_01598, AB901B6_01025 and AB945B12_00993, respectively.

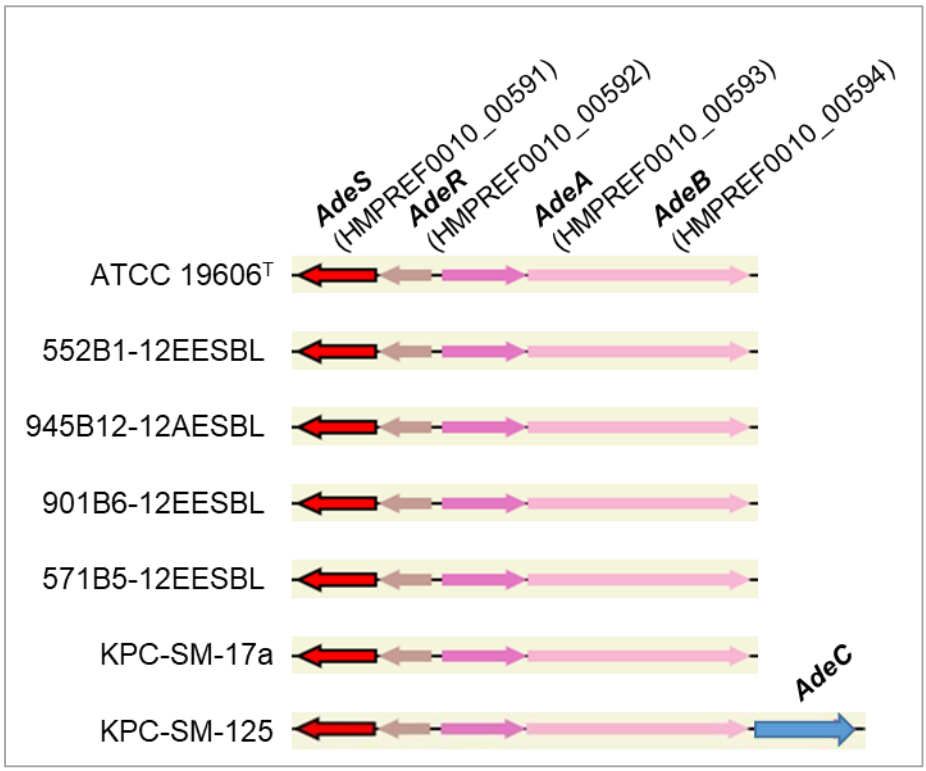

Figure S8. Distribution of genes encoding two-component regulatory system (AdeRS) and AdeABC efflux pumps in the genomes of ATCC 19606^T (ACQB00000000) and *A. baumannii* strains. *AdeC* gene was only present in stain KPC-SM-125. Lack of insertion of IS*Aba1* in *adeS* gene of all strains. Horizontal arrows indicate the direction and orientation of the gene. Locus tag numbers were given in brackets.

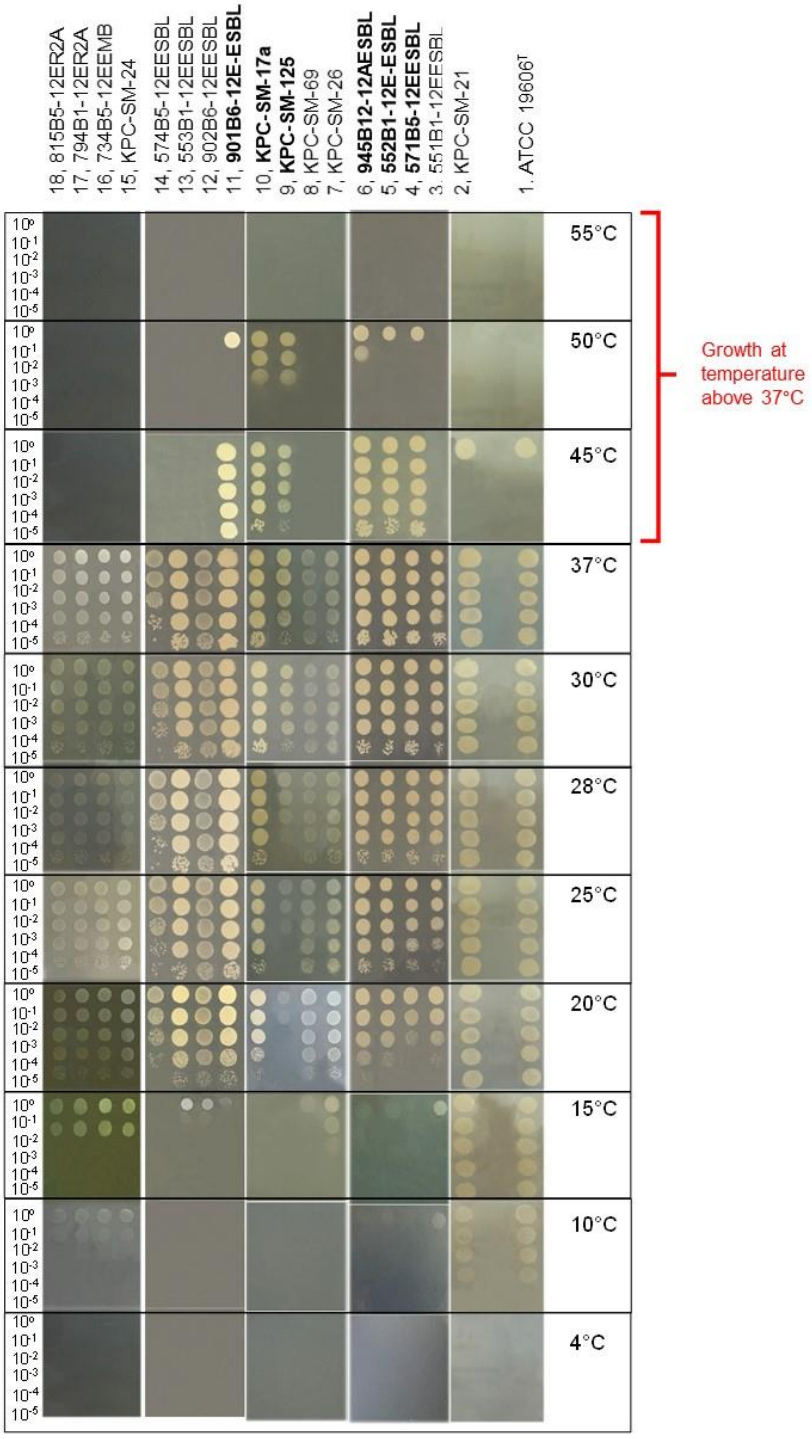

Figure S9. Spot assay test for temperature dependent growth for *A. baumannii* ATCC 19606^{T} and 17 *Acinetobacter* sp. isolates: Strains related to *A. baumannii* are represented in bold font.

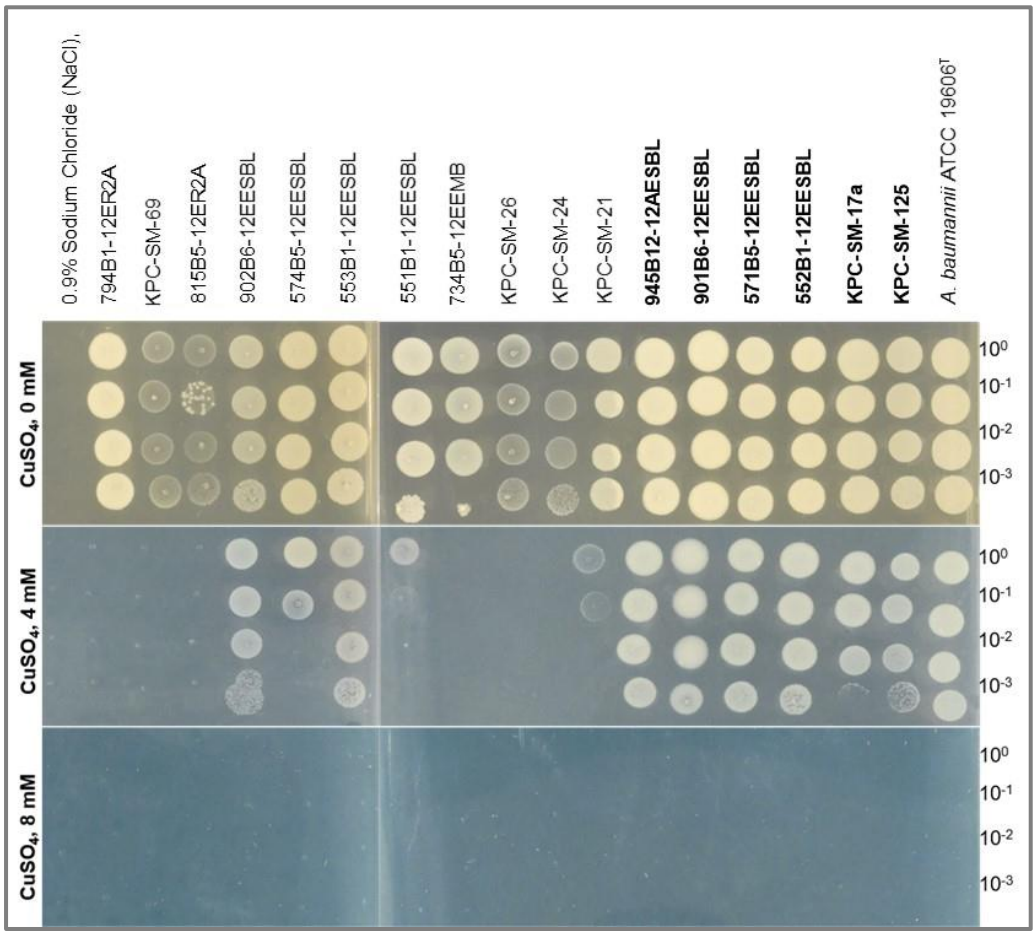

Figure S10. Copper tolerance was tested by pipetting 5 µL of serial dilution (10^0 – 10^{-3} dilution, turbidity adjusted to standard 0.5 McFarland) of overnight cultured isolates in Muller Hinton agar plates (ROTH) supplemented with (4, 8, 12, 16, 20, 24, 32 and 36 mM, adjusted to pH 7.2) $CuSO_4$ $5H_2O$ (MERCK). The plates were incubated at 37°C aerobically, and checked for growth after 24h of incubation. The lowest copper concentration that suppressed growth was considered as the MIC value. The growth was completely suppressed in concentration of 8 mM and above (data not shown). Strains related to *A. baumannii* are represented in bold font.

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Annex

Chapter IV, Supporting Information

Genomic plasticity and adaptive capacity of the quaternary alkyl-ammonium compound and copper tolerant *Acinetobacter bohemicus* strain QAC-21b isolated from pig manure

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Supplementary tables

Table S1. Minimum inhibitory concentration (MIC) values of strain QAC-21b against QAACs and antibiotics given in µg mL-1. MIC value assigned to S, Susceptible, I, Intermediate, or R, Resistant according to CLSI or EUCAST database. -, no data available.

Tested compounds	***A. bohemicus* QAC-21b MIC [µg ml^{-1}]**	***A. bohemicus* QAC-21b S, I, R assignment according to CLSI**
QAACs		
BAC-C12	50	-
DADMAC-C10	2.5	-
Antibiotics		
Amikacin	<0.5	S
Cefotaxime	2	S
Ceftazidime	2	S
Ceftazidime/3-APB	2	-
Ceftazidime/Avibactam	<1/4	-
Ceftolozane/Tazobactam	<1/4	-
Chloramphenicol	<8	-
Ciprofloxacin	<0.25	S
Colistin	<1	S
Fosfomycin	>64	-
Imipenem	<1	S
Levofloxacin	<0.5	S
Meropenem	<0.125	S
Meropenem/3-APB	0.5	-
Meropenem/EDTA	<0.25	-
Piperacillin	<8	S
Piperacillin/Tazobactam	<4/4	S
Temocillin	<32	-
Tigecycline	<0.25	-
Trimethoprim/Sulfamethoxazole	<1/9	S
Amoxicillin	<0.5	-
Oxacillin	16	-
Ceftiofur	8	-
Ceftiofur/clavulanic acid	<0.25/4	-
Cefquinom	0.5	-
Cefquinom/clavulanic acid	<0.25/4	-
Enrofloxacin	<0.0625	-
Tetracycline	8	I
Tylosin	>16	-
Florfenicol	8	-
Sulfamethoxazole	<4	-

Table S2. Comparative genomics of QAC-21b with next related type strains of *Acinetobacter* species and *A. baumannii* strains including the type strain and environmental and clinical strains

Strains	***A. bohemicus* QAC-21b**	***A. bohemicus* ANC 3994^T**	***A. bohemicus* KCTC 42081**	***A. johnsonii* CIP 64.6^T**	***A. kookii* KCTC 32033^T**	***A. baumannii* ATCC 17978**	***A. baumannii* AYE**	***A. baumannii* KPC-SM-125**	***A. baumannii* ATCC 19606^T**
Isolation source	Pig manure	Deciduous forest soil (Czech Republic)	Textile dyeing wastewater treatment pond Pakistan:Islamabad, Industrial area, Kohnoor mill bioremediation pond	Human duodenum	Soil	Human cerebrospinal fluid	Patient with urinary tract infection	Biogas plant digestate	Urine
Reference	This study	Krizova et al. (2014)	Abbas et al. (2014)	Bovet and Grimont (1986)	Choi et al. (2013)	Bovet and Grimont, 1986	Poirel et al. (2003)	Pulami et al. (2020)	Bovet and Grimont (1986)
NCBI Genome accession number	NZ_CAJJDZ000000000	NZ_APOH00000000	NZ_FOZU00000000	NZ_APON00000000	NZ_FMYO00000000	CP000521.1	NC_010410.1	NZ_CACSGU000000000	NZ_ACQB00000000
Genetic resistance determinants (Accession number)									
Efflux pumps genes (Accession number)									
SMR family efflux QacEΔ1 (QAACs efflux pump)	Absent	Absent	Absent	Absent	Absent	Absent	*qacEΔ1* (CT025832)	Absent	Absent

Multidrug and toxic compound extrusion (MATE) family efflux AbeM (QAACs, dyes and antibiotic efflux pumps)	*abeM (mdtK)* (QAC21B_02006)	*abeM* (F994_00296)	*abeM* (SAMN05444586_1006117)	*abeM* (F986_03240)	*abeM* (SAMN05421732_10810)	*abeM* (A1S_0395)	*abeM* (ABAYE3381)	*abeM* (ABKPCSM125_00210)	*abeM* (HMPREF0010_01847)
Small multidrug resistance (SMR) family efflux AbeS (QAACs, dyes and antibiotic efflux pumps)	*abeS (emrE)* (QAC21B_01577)	*abeS* (F994_02286)	*abeS* (SAMN05444586_100969)	*abeS* (F986_00511)	*abeS* (SAMN05421732_101897)	*abeS* (A1S_2298)	*abeS* (ABAYE1181)	*abeS* (ABKPCSM125_01695)	*abeS* (HMPREF0010_00032)
SMR family efflux SugE (QAACs and dyes efflux pumps)	*sugE* (QAC21B_03254)	*sugE* (F994_00697)	*sugE* (SAMN05444586_101190)	*sugE* (F986_01553)	*sugE* (SAMN05421732_101897)	*sugE* (A1S_0710)	*sugE* (ABAYE3107)	*sugE* (ABKPCSM125_03091)	*sugE* (HMPREF0010_01213)
Resistance nodulation division (RND) type efflux AdeIJK (QAACs, dyes and antibiotic efflux pump)	*adeIJK* (*oprM*) [(QAC21B_01138 to,_01140)	*adeIJK* (F994_00903 to _00905)	*adeIJK* (SAMN05444586_100846 to _100848)	*adeIJK* (F986_01786 to _01788)	*adeIJK* (SAMN05421732_10556 to _10558)	*adeIJK* (A1S_2735 to _2737)	*adeIJK* (ABAYE0746 to ABAYE0748)	*adeIJK* (ABKPCSM125_00755 to _00757)	*adeIJK* (HMPREF0010_02880 to _02882)
RND type efflux AdeABC and AdeRS (antibiotic efflux pump)	Absent	Absent	Absent	Absent	Absent	*adeAB*(A1S_1750 to 1754), AdeRS ()	*adeABC* (ABAYE1821 to ABAYE1823), *adeRS* (ABAYE1819 to ABAYE1820)	*adeABC* (ABKPCSM125_02330 to _02332), *adeRS* (ABKPCSM125_02328 to _02329)	*adeAB* (HMPREF0010_00593 to _00594), *adeRS* (HMPREF0010_00591 to _00592),
Tellurium resistance protein TerZ [Tellurium (Te)]	*terZ* (QAC21B_02260)	*terZ* (F994_02978)	*terZ* (SAMN05444586_10109)	Absent	Absent	Absent	Absent	Absent	Absent
Tellurium resistance protein TerE [Tellurium (Te)]	*terE* (QAC21B_02263)	*terE* (F994_02981)	*terE* (SAMN05444586_101012)	Absent	Absent	Absent	Absent	Absent	Absent

Tellurium resistance protein TerD [Tellurium (Te)]	*terD* (QAC21B_02265)	*terD* (F994_02983)	*terD* (SAMN05444586_101014)	Absent	Absent	Absent	Absent	Absent	Absent
Tellurium resistance protein TerA [Tellurium (Te)]	*terA* (QAC21B_02266)	*terA* (F994_02984)	*terA* (SAMN05444586_101015)	Absent	Absent	Absent	Absent	Absent	Absent
ATP-dependent DNA helicase RuvB [Tellurium (Te)/Selenium (Se)]	*ruvB* (QAC21B_00133)	*ruvB* (F994_01894)	*ruvB* (SAMN05444586_101477)	*ruvB* (F986_00963)	*ruvB* (SAMN05421732_101574)	*ruvB* (A1S_2588)	*ruvB* (ABAYE0909)	*ruvB* (ABKPCSM125_01441)	*ruvB* (HMPREF0010_02149)
CorA metal ion transporter (MIT) family [Magnesium (Mg), Cobalt (Co), Nickel (Ni) and Manganese (Mn)]	*corA* (QAC21B_02480)	*corA* (F994_00277)	*corA* (SAMN05444586_10137)	*corA* (F986_03218)	*corA* (SAMN05421732_11310)	*corA* (A1S_3098)	*corA* (ABAYE0390)	*corA* (ABKPCSM125_03558)	*corA* (HMPREF0010_02609)
Arsenate reductase ArsC [Arsenic (As)]	*arsC* (QAC21B_02465)	*arsC* (F994_00973)	*arsC* (SAMN05444586_102917)	*arsC* (F986_01391)	*arsC* (SAMN05421732_10480)	*arsC* (A1S_1452)	*arsC* (ABAYE2200)	*arsC* (ABKPCSM125_02791)	(HMPREF0010_00924)
Arsenite resistance protein ArsB [Arsenic (As)]	*arsB* (QAC21B_02468)	*arsB* (F994_00976)	*arsB* (SAMN05444586_102914)	*arsB* (F986_01388)	*arsB* (SAMN05421732_10475)	*arsB* (A1S_1454)	*arsB* (ABAYE2198)	*arsB* (ABKPCSM125_02793)	(HMPREF0010_00922)
Cobalt (Co), Zinc (Zn), Cadmium (Cd) efflux pumps	*czcA* (QAC21B_03355)	*czcA* (F994_01136)	*czcA* (SAMN05444586_104711)	*czcA* (F986_00575)	Absent	*czcA* (A1S_3217)	*czcA* (ABAYE0271)	*czcA* (ABKPCSM125_03438)	*czcA* (HMPREF0010_02496)
Cobalt (Co) and Zinc (Zn) efflux pumps	*czcD* (QAC21B_03356)	*czcD* (F994_00374)	*czcD* (SAMN05444586_104712)	*czcD* (F986_00579)	*czcD* (SAMN05421732_10894)	*czcD* (A1S_3214)	*czcD* (ABAYE0272)	*czcD* (ABKPCSM125_03439)	*czcD* (HMPREF0010_02497)
Copper resistance protein (PcoAB, CopA, CopB, CopC, CopD) [Copper (Cu)]	*pcoAB* (QAC21B_03255 to _03256), *copC* (QAC21B_03822), *copD*	*pcoAB* (F994_00695 to _00696), *copC* (Absent), *copD*	*pcoAB* (SAMN05444586_101191 to _101192), *copC* (SAMN05444586_10522),	*pcoAB* (Absent), *copA* (F986_01556), *copB* (F986_01	*pcoAB* (SAMN05421732_101898 to _101899), *copD* (Absent),	*pcoAB* (A1S_0707 to _0708), *copC* (A1S_2940), *copD* (A1S_2941)	*pcoAB* (ABAYE3110 to ABAYE3111), *copC* (ABAYE3206), *copD*	*pcoAB* (ABKPCSM125_03093 to _03094), *copD* (Absent), *copC*	*pcoAB* (HMPREF0010_01210 to _01211), *copA* (Absent), *copB*

	(QAC21B_03821), *copA* (QAC21B_03343), *copB* (QAC21B_03344)	(Absent), *copA* (Absent), *copB* (Absent)	*copD* (SAMN05444586_10521), *copA* [(SAMN05444586_105211), *copB* (SAMN05444586_105212)	975), *copD* (F986_01962), *copC* (F986_01963)	*copC* (Absent), *copA* (Absent), *copB* (Absent)	, *copB* (A1S_2935), *copA* (A1S_2936)	(ABAYE3207), *copB* (ABAYE3200), *copA* (ABAYE3201)	(Absent), *copA* (Absent), *copB* (Absent)	(Absent), *copC* (Absent), *copD* (Absent)
Further resistance determinants									
Class D type intrinsic β-lactamse (intrinsic)	*bla*$_{OXA-133}$ (QAC21B_02624)	*bla*$_{OXA-296}$ (F994_00492)	Frameshifted; internal stop codon (SAMN05444586_104315 and 16)*	*bla*$_{OXA-281}$ (F986_02727)	Absent	*bla*$_{OXA-95}$ (A1S_1517)	*bla*$_{OXA-69}$ (ABAYE2122)	*bla*$_{OXA-909}$ (ABKPCSM125_02591)	*bla*$_{OXA-98}$ (HMPREF0010_00844)
Insertion sequence (IS) element									
Putative IS element 1									
Locus tag number	(QAC21B_03920)	Absent	Absent	(F986_01630)	Absent	Absent	Absent	Absent	Absent
DNA similarity	94% to IS*Aba14* (IS family: IS3, origin: *A. baumannii*)								
Amino acids similarity	97% to IS*Aba14*								
Coverage	54% ORF of IS*Aba14*								
Putative IS element 2									
Locus tag number	(QAC21B_03923)	Absent	(SAMN05444586_11041)	(F986_00702)	Absent	(A1S_0628)	Absent	Absent	Absent

DNA similarity	99% to IS*Aba12* (IS family: IS5, origin: *A. baumannii*)								
Amino acids similarity	98% to IS*Aba12*								
Coverage	100% ORF of IS*Aba12*								
Putative IS element 3									
Locus tag number	(QAC21B_03925)	(F994_02663)	Absent	(F986_00558)	Absent	Absent	Absent	Absent	Absent
DNA similarity	99% to IS*Alw3* (IS family: IS1, origin: *A. lwoffii*)								
Amino acids similarity	98% to IS*Alw3*								
Coverage	100% ORF of IS*Alw3*								
Type 6 secretion system (T6SS) linked genes and other virulence factors									
Hemolysin coregulated protein (Hcp)	*hcp* (QAC21B_03466)	*hcp* (F994_02474)	*hcp* (SAMN05444586_100437)	*hcp* (F986_00533)	*hcp* (SAMN05421732_10124)	*hcp* (A1S_1296)	*hcp* (ABAYE2413)	*hcp* (ABKPCSM125_02612)	*hcp* (HMPREF0010_01123)
Valine-glycine repeat protein G (VgrG)	*vgrG* (QAC21B_02199)	*vgrG* (F994_02954)	*vgrG*, Frameshifted; internal stop codon (SAMN05444586_102840)	*vgrG* (F986_02152)	*vgrG* (SAMN05421732_10733)	*vgrG* (A1S_3364)	*vgrG* (ABAYE0118)	*vgrG* (ABKPCSM125_01237)	*vgrG* (HMPREF0010_03005)

Membrane spanning complex protein TssM	*tssM* (QAC21B_03461)	*tssM* (F994_02479)	*tssM* (SAMN05444586_100442)	*tssM* (F986_00538)	*tssM* (SAMN05421732_10119)	*tssM* (A1S_1302)	*tssM* (ABAYE2408)	*tssM* (ABKPCSM125_02617)	*tssM* (HMPREF0010_01118)
Baseplate components T6SS protein TssE	*tssE* (QAC21B_03465)	*tssE* (F994_02475)	*tssE* (SAMN05444586_100438)	*tssE* (F986_00534)	*tssE* (SAMN05421732_10123)	*tssE* (A1S_1297)	*tssE* (ABAYE2412)	*tssE* (ABKPCSM125_02613)	*tssE* (HMPREF0010_01122)
Baseplate components T6SS protein TssF	*tssF* (QAC21B_03464)	*tssF* (F994_02476)	*tssF* (SAMN05444586_100439)	*tssF* (F986_00535)	*tssF* (SAMN05421732_10122)	*tssF* (A1S_1299)	*tssF* (ABAYE2411)	*tssF* (ABKPCSM125_02614)	*tssF* (HMPREF0010_01121)
Baseplate components T6SS protein TssG	*tssG* (QAC21B_03463)	*tssG* (F994_02477)	*tssG* (SAMN05444586_100440)	*tssG* (F986_00536)	*tssG* (SAMN05421732_10121)	*tssG* (A1S_1300)	*tssG* (ABAYE2410)	*tssG* (ABKPCSM125_02615)	*tssG* (HMPREF0010_01120)
Sheath components T6SS protein TssB	*tssB* (QAC21B_03468)	*tssB* (F994_02472)	*tssB* (SAMN05444586_100435)	*tssB* (F986_00531)	*tssB* (SAMN05421732_10126)	*tssB* (A1S_1294)	*tssB* (ABAYE2415)	*tssB* (ABKPCSM125_02610)	*tssB* (HMPREF0010_01125)
Sheath components T6SS protein TssC	*tssC* (QAC21B_03467)	*tssC* (F994_02473)	*tssC* (SAMN05444586_100436)	*tssC* (F986_00532)	*tssC* (SAMN05421732_10125)	*tssC* (A1S_1295)	*tssC* (ABAYE2414)	*tssC* (ABKPCSM125_02611)	*tssC* (HMPREF0010_01124)
Membrane associated protein for reduced permeability (intrinsic OmpA)	*ompA* [(QAC21B_03084)	*ompA* (F994_01549)	*ompA* (SAMN05444586_101243)	*ompA* (F986_01636)	*ompA* (SAMN05421732_11053)	*ompA* (A1S_2840)	*ompA* (ABAYE0640)	*ompA* (ABKPCSM125_00647)	*ompA* (HMPREF0010_02782)
Phospholipase D (PlcD)	*plcD* (QAC21B_03108)	*plcD* (F994_01528)	*plcD* (SAMN05444586_101221)	*plcD* (F986_01612)	*plcD* (SAMN05421732_11074)	*plcD* (A1S_2989)	*plcD* (ABAYE0498)	*plcD* (ABKPCSM125_03038)	*plcD* (HMPREF0010_03706)
Two component response regulator transcription factor (BmfRS)	*bmfRS* (QAC21B_02363 to _02364)	*bmfRS* (F994_00745 to _00746)	*bmfRS* (SAMN05444586_101142 to _101143)	*bmfRS* (F986_01756 to _01757)	*bmfRS* (SAMN05421732_101839 to _101840)	*bmfRS* (A1S_0748 to _0749)	*bmfRS* (ABAYE3063 to ABAYE3064)	*bmfRS* (ABKPCSM125_00534 to _00535)	*bmfRS* (HMPREF0010_01249 to _01250)

Table S3. Potential phage genes of *A. bohemicus* QAC-21b. Contig sequences were examined for phage related genes using PHASTER (https://phaster.ca/; Zhou et al. 2011; Arndt et al. 2016).

No.	Contig Accession	Region length	Completeness	Score	Position	Most common phage	Phage accession	GC (%) content
1	NZ_CAJJDZ010000001	37.5 kb	**Intact**	150	59520-97070	PHAGE_Mannhe_vB_MhM_3927 AP2	NC_028766	41.89%
2	NZ_CAJJDZ010000001	21.2 kb	**Intact**	140	233340-254552	PHAGE_Burkho_KS14	NC_015273	40.32%
3	NZ_CAJJDZ010000001	5.6 kb	Incomplete	30	622985-628631	PHAGE_Pseudo_H66	NC_042342	36.28%
4	NZ_CAJJDZ010000002	21.2 kb	**Intact**	140	104633-125845	PHAGE_Burkho_KS14	NC_015273	40.32%
5	NZ_CAJJDZ010000002	32 kb	**Intact**	140	806480-838588	PHAGE_Acinet_YMC11/11/R317 7	NC_041866	39.63%
6	NZ_CAJJDZ010000003	31.6 kb	Incomplete	60	140679-172329	PHAGE_Acinet_vB_AbaS_TRS1	NC_031098	38.19%
7	NZ_CAJJDZ010000004	7.1 kb	Incomplete	50	13960-21133	PHAGE_Salini_M8CC_19	NC_042349	39.95%
8	NZ_CAJJDZ010000007	32.2 kb	**Intact**	110	38060-70261	PHAGE_Acinet_YMC11/11/R317 7	NC_041866	40.60%
9	NZ_CAJJDZ010000010	18.1 kb	Questionable	80	22213-40401	PHAGE_Acinet_Bphi_B1251	NC_019541	40.98%
10	NZ_CAJJDZ010000010	40.2 kb	Questionable	80	74140-114354	PHAGE_Stenot_S1	NC_011589	37.47%
11	NZ_CAJJDZ010000010	18 kb	Questionable	70	116691-134719	PHAGE_Stx2_II	NC_004914	38.05%
12	NZ_CAJJDZ010000010	20.2 kb	Incomplete	60	139167-159455	PHAGE_Stx2_c_1717	NC_011357	39.03%
13	NZ_CAJJDZ010000014	11.3 kb	Incomplete	60	3251-14616	PHAGE_Escher_RCS47	NC_042128	39.16%

Table S4. Potential phage genes of *A. bohemicus* ANC 3994^{T}. Contig sequences were examined for phage related genes using PHASTER.

No.	Contig Accession	Region length	Completeness	Score	Position	Most common phage	Phage accession	GC (%) content
1	NZ_APOH01000003	6.9 kb	Incomplete	10	22190-29111	PHAGE_Escher_phAPEC8	NC_020079	35.09%
2	NZ_APOH01000003	9.5 kb	Incomplete	30	89712-99284	PHAGE_Gordon_Schwabeltier	NC_031255	43.16%
3	NZ_APOH01000010	8.2 kb	Incomplete	10	73071-81279	PHAGE_Tenaci_pT24	NC_049383	41.92%
4	NZ_APOH01000015	21.2 kb	**Intact**	130	205705-226951	PHAGE_Burkho_KS14	NC_015273	39.75%
5	NZ_APOH01000017	22.7 kb	Questionable	70	32423-55137	PHAGE_Escher_RCS47	NC_042128	36.61%
6	NZ_APOH01000021	30.6 kb	Questionable	80	19277-49889	PHAGE_Pseudo_F116	NC_006552	39.93%
7	NZ_APOH01000023	11.7 kb	Incomplete	50	20537-32297	PHAGE_Acinet_vB_AbaM_ME3	NC_041884	38.09%
8	NZ_APOH01000025	9.4 kb	Incomplete	10	8572-18026	PHAGE_Bacill_SP_15	NC_031245	39.79%

Table S5. Potential phage genes of *A. bohemicus* KCTC 42081. Contig sequences were examined for phage related genes using PHASTER.

No.	Contig Accession	Region length	Completeness	Score	Position	Most common phage	Phage accession	GC (%) content
1	NZ_FOZU01000016	24.9 Kb	Incomplete	40	7425-32334	PHAGE_Shigel_Sf6	NC_005344	37.53%
2	NZ_FOZU01000020	5.3Kb	Incomplete	10	8977-14341	PHAGE_Acinet_Bphi_B1251	NC_019541	36.05%
3	NZ_FOZU01000029	21.7Kb	Incomplete	30	9074-30841	PHAGE_Escher_RCS47	NC_042128	40.57%
4	NZ_FOZU01000055	8.6Kb	Incomplete	40	1762-10404	PHAGE_Acinet_vB_AbaS_TRS1	NC_031098	40.53%

Table S6. List of genes associated to metabolism, transposon, IS elements, virulence, metal efflux pumps and antibiotic resistance present within putative genomic islands (GIs) of strain QAC-21b, [exception: genes encoding hypothetical proteins and other functional genes present in the putative GIs were not listed]. The GIs were predicted using IslandViewer4 (Bertelli et al. 2017).

				Putative GIs region in the genome predicted by IslandViewer4	
Strain	Gene	Product	Locus tag	Start	End
Acinetobacter bohemicus strain QAC-21b	*czcA*	Cobalt-zinc-cadmium resistance protein CzcA	QAC21B_03355	59,599	150,270
	czcD	Metal cation efflux system protein CzcD	QAC21B_03356		
	czcO	Putative oxidoreductase CzcO	QAC21B_03347		
	feoB	Ferrous iron transport protein B homolog	QAC21B_03348		
	pcoA	Copper resistance protein A	QAC21B_03343		
	insF	Transposase InsF for insertion sequence IS3	QAC21B_03328		
	vapB	Virulence-associated protein B	QAC21B_03319		
	tnp1	Transposase for insertion sequence-like element IS431mec	QAC21B_03318		
	tnpR	Transposon Tn501 resolvase	QAC21B_03300		
	tnpA	Transposase	QAC21B_02671	429,804	548,856
		Tellurite resistance protein	QAC21B_02636		
		Putative transposase for transposon Tn903	QAC21B_02464	717,902	724,817
	mdtB	Multidrug resistance protein MdtB	QAC21B_03217	2,449,583	2,459,341
	mdtC	Multidrug resistance protein MdtC	QAC21B_03216		
	insF	Transposase InsF for insertion sequence IS3	QAC21B_03130	2,555,928	2,561,196
	tnpA1	TnpA-A	QAC21B_03758	2,722,017	2,756,199
	tnp2	Transposase for insertion sequence-like element IS431mec	QAC21B_03765		
	int5	Integrase	QAC21B_03774		
	vapB	Virulence-associated protein B	QAC21B_03784		
	tnpR	Transposon Tn501 resolvase	QAC21B_03812	2,788,199	2,797,179
	insK	Putative transposase InsK for insertion sequence element IS150	QAC21B_03815		
	tnpA	TnpA-A	QAC21B_00636	3,672,146	3,683,410
	tnp1	Transposase for insertion sequence-like element IS431mec	QAC21B_03607	3,952,839	3,992,385
	tnpR	Transposon Tn501 resolvase	QAC21B_03606		

	macB	Macrolide export ATP-binding/permease protein MacB	QAC21B_03623		
	IS*401*	Insertion element IS401 uncharacterized 12,4 kDa protein	QAC21B_03627		
	tnp1008	Transposase	QAC21B_03901		
	tnp1	Transposase for insertion sequence-like element IS431mec	QAC21B_03905		
Acinetobacter bohemicus ANC 3994^T		IS605 OrfB family transposase	F994_02674	2,075,536	2,122,615
Acinetobacter pakistanensis ANC 5076 = KCTC 42081^T		MBL superfamily protein	SAMN05444586_1003173	299,813	315,463
		Acetyl esterase/lipase	SAMN05444586_100614	1,004,645	1,044,425
		Transposase IS6 family	SAMN05444586_104714		
	czcD	Cobalt-zinc-cadmium efflux system protein	SAMN05444586_104712		
	czcA	Cobalt-zinc-cadmium resistance protein CzcA	SAMN05444586_104711		
	feoA	Ferrous iron transport protein A	SAMN05444586_10475		
	feoB	Ferrous iron transport protein B	SAMN05444586_10474		
	czcO	Predicted flavoprotein CzcO associated with the cation diffusion facilitator CzcD	SAMN05444586_10472		
	arsH	Arsenical resistance protein ArsH	SAMN05444586_10471		
		Predicted arabinose efflux permease, MFS family	SAMN05444586_10435		
		Putative transposase	SAMN05444586_101865	1,263,313	1,271,902
	tniB	TniB protein	SAMN05444586_101866		
	tniQ	TniQ protein	SAMN05444586_101868		
		Putative transposase	SAMN05444586_10108	1,382,063	1,401,923
		Transposase IS6 family	SAMN05444586_102936		
		OmpA family protein	SAMN05444586_10289	1,441,629	1,450,983

		Glutamin-(asparagin-)ase	SAMN05444586_101621	1,598,673	1,633,702
		Integrase	SAMN05444586_102069	2,993,905	3,010,214
	cusS	Two-component system, OmpR family, heavy metal sensor histidine kinase CusS	SAMN05444586_10528	3,428,660	3,507,668
	cusR	Two-component system, OmpR family, copper resistance phosphate regulon response regulator CusR	SAMN05444586_10529		
	copA	Copper resistance protein A	SAMN05444586_105211		
	copB	Copper resistance protein B	SAMN05444586_105212		
		Transposase IS6 family	SAMN05444586_10982		
		Transposase	SAMN05444586_10961		
		Transposase	SAMN05444586_10515		
		Transposase	SAMN05444586_103011	3,516,260	3,559,379
		Transposase	SAMN05444586_104110	3,577,878	3,745,643
		Transposase	SAMN05444586_104120		
		Transposase	SAMN05444586_10912		
		Transposase IS6 family	SAMN05444586_10815		
		Transposase IS6 family	SAMN05444586_10891		
		Integrase	SAMN05444586_10655		
		Transposase	SAMN05444586_10656		
		Transposase	SAMN05444586_10607		
		Transposase	SAMN05444586_10734	3,809,907	3,826,362
		Transposase	SAMN05444586_10821		
		Transposase IS66 family	SAMN05444586_10823		
		Transposase IS630 family	SAMN05444586_11021		
	mdtC	Multidrug efflux pump MdtC	SAMN05444586_100488	2,485,149	2,496,314
	mdtB	multidrug efflux pump MdtB	SAMN05444586_100489		
		Transposase IS6 family	SAMN05444586_10541	2,586,374	2,592,167

Supplementary figures

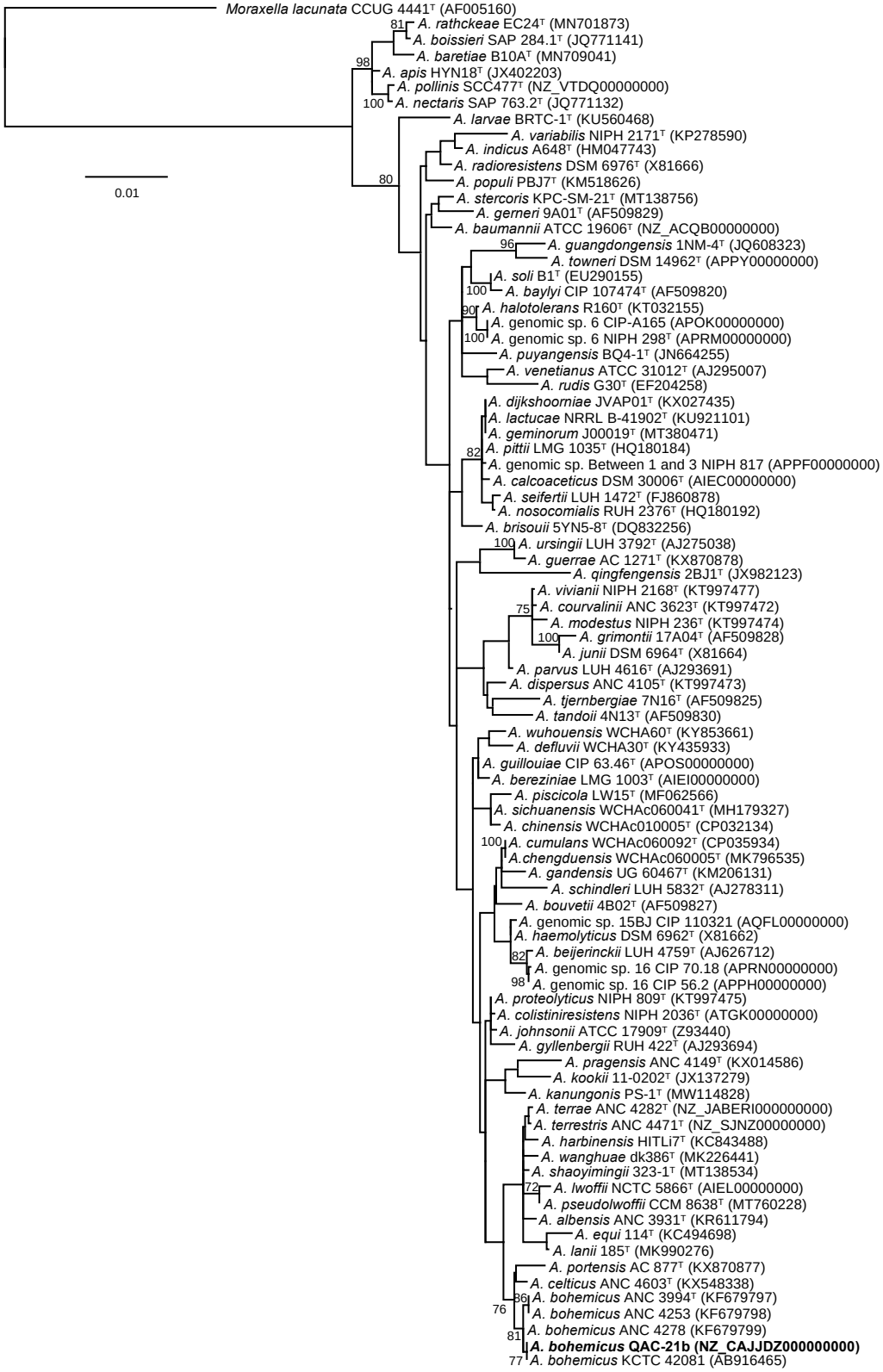

Figure S1. Phylogenetic placement of QAC-21b into a distinct cluster with strains of the species *A. bohemicus*. The phylogenetic tree was based on nearly full length nucleotide sequence of 16S rRNA gene. The evolutionary history was inferred by

using the Maximum Likelihood method (Felsenstein 1981) based on the Kimura 2-parameter model (Kimura 1980) using a discrete gamma distribution to model evolutionary rate differences among sites [5 categories (+*G*)] and assuming some sites to be evolutionarily invariable ([+*I*]. The bootstrap values (≥ 70%) based on 100 re-samplings are shown above the branches. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. Scale, number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Analysis was according to Pulami et al. (2021). Bar, 0.01 nucleotide substitutions per nucleotide position.

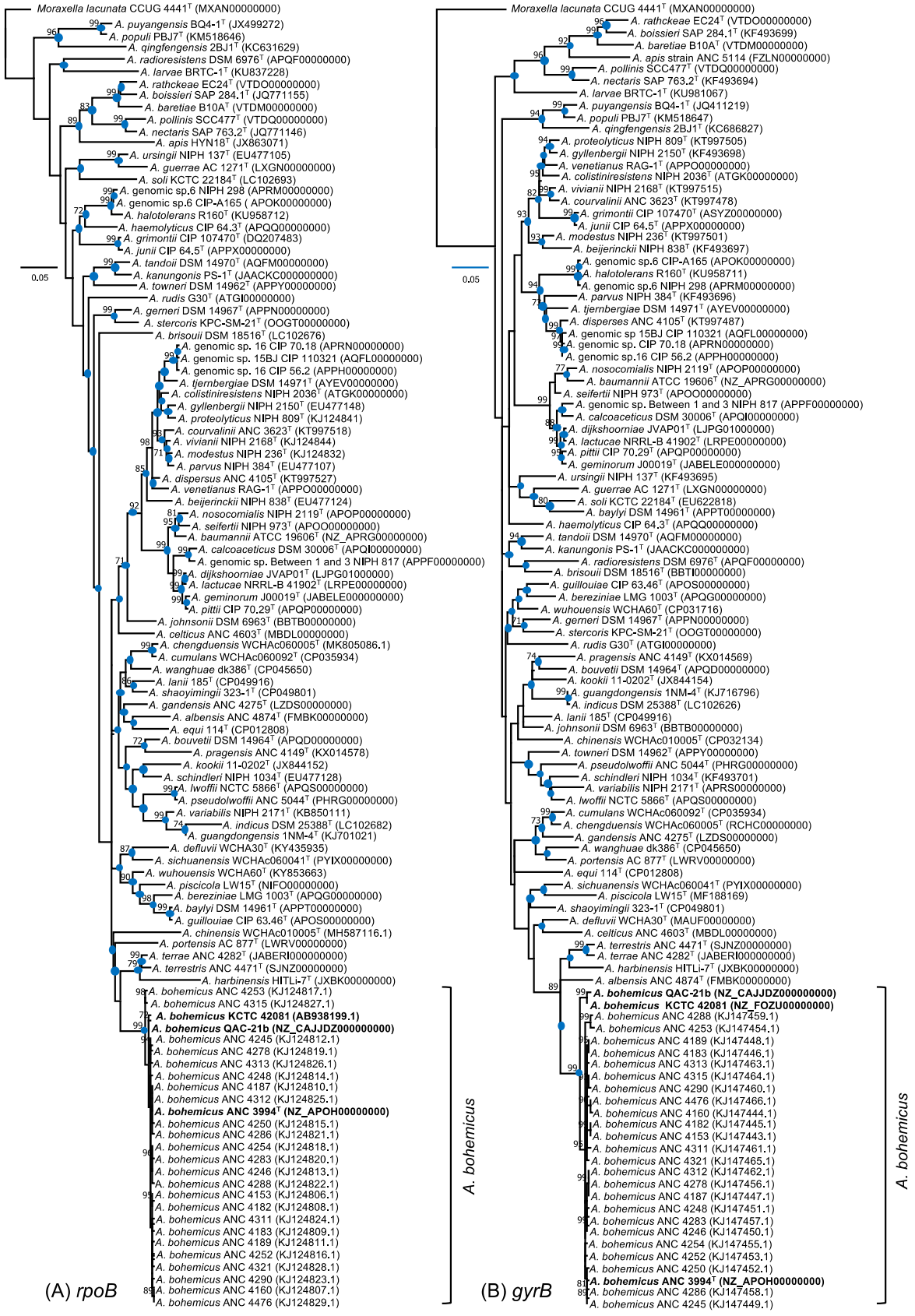

Figure S2. Phylogenetic placement of *A. bohemicus* QAC-21b into the species cluster of *A. bohemicus* within the genus *Acinetobacter*. Phylogenies calculated by Maximum

Likelihood method (Felsenstein 1981) based on the General Time Reversible model (GTR; Nei and Kumar 2000) with a discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G)] assuming some sites to be evolutionarily invariable (+I). Phylogenetic trees of (A) *rpoB* and (B) *gyrB* were based on total of 856 and 621 nucleotide positions in the final datasets. All positions containing gaps and missing data were eliminated. Bootstrap values (≥70%) after 100 resamplings are indicated at branch nodes; bar, number of substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Circle represented nodes also present in phylogenetic tree calculated with the Neighbor-joining method (Saitou and Nei 1987)

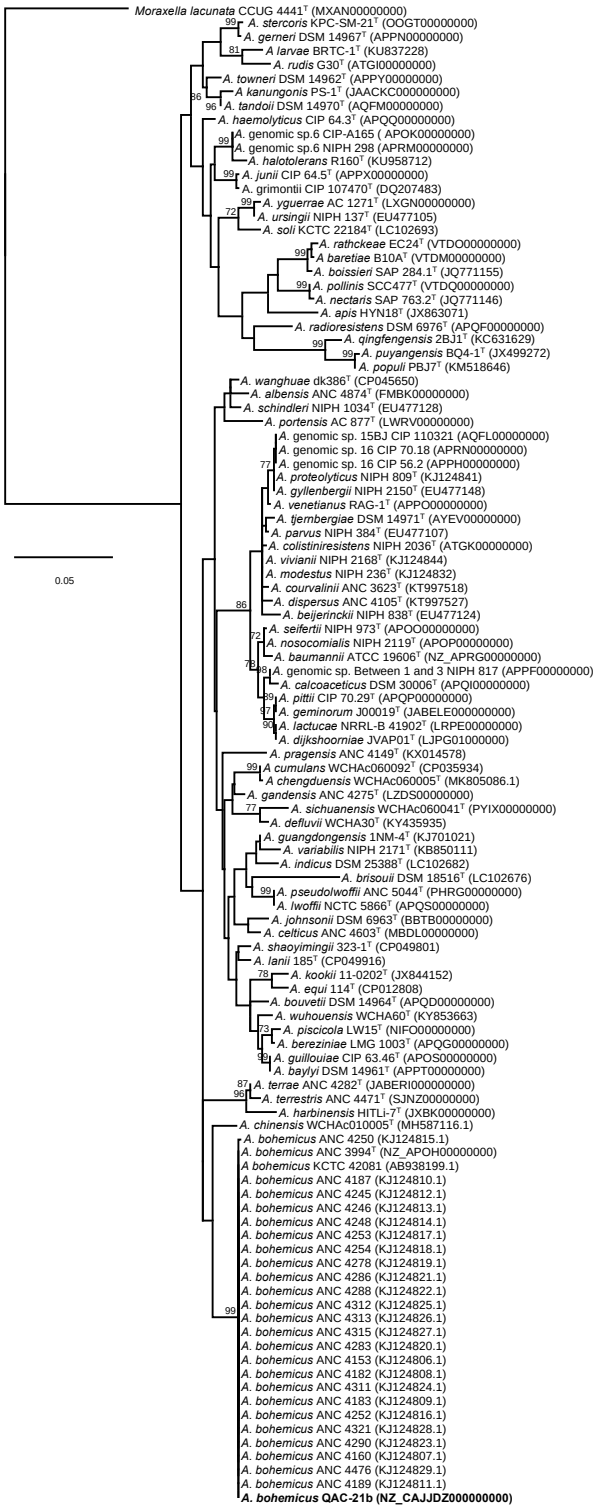

Figure S3. Phylogenetic tree based on partial amino acid sequence of *rpoB* showing the placement of QAC-21b into the species cluster of *A. bohemicus*. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992) applying a discrete Gamma distribution to model evolutionary rate differences among sites (+G) assuming some sites to be

evolutionarily invariable (+I). Bootstrap values (≥70%) based on 100 re-samplings are shown above the branches. The analysis involved total of 284 positions in the final dataset. Tree was calculated in MEGA7. Bar, rate of substitution per amino acid position.

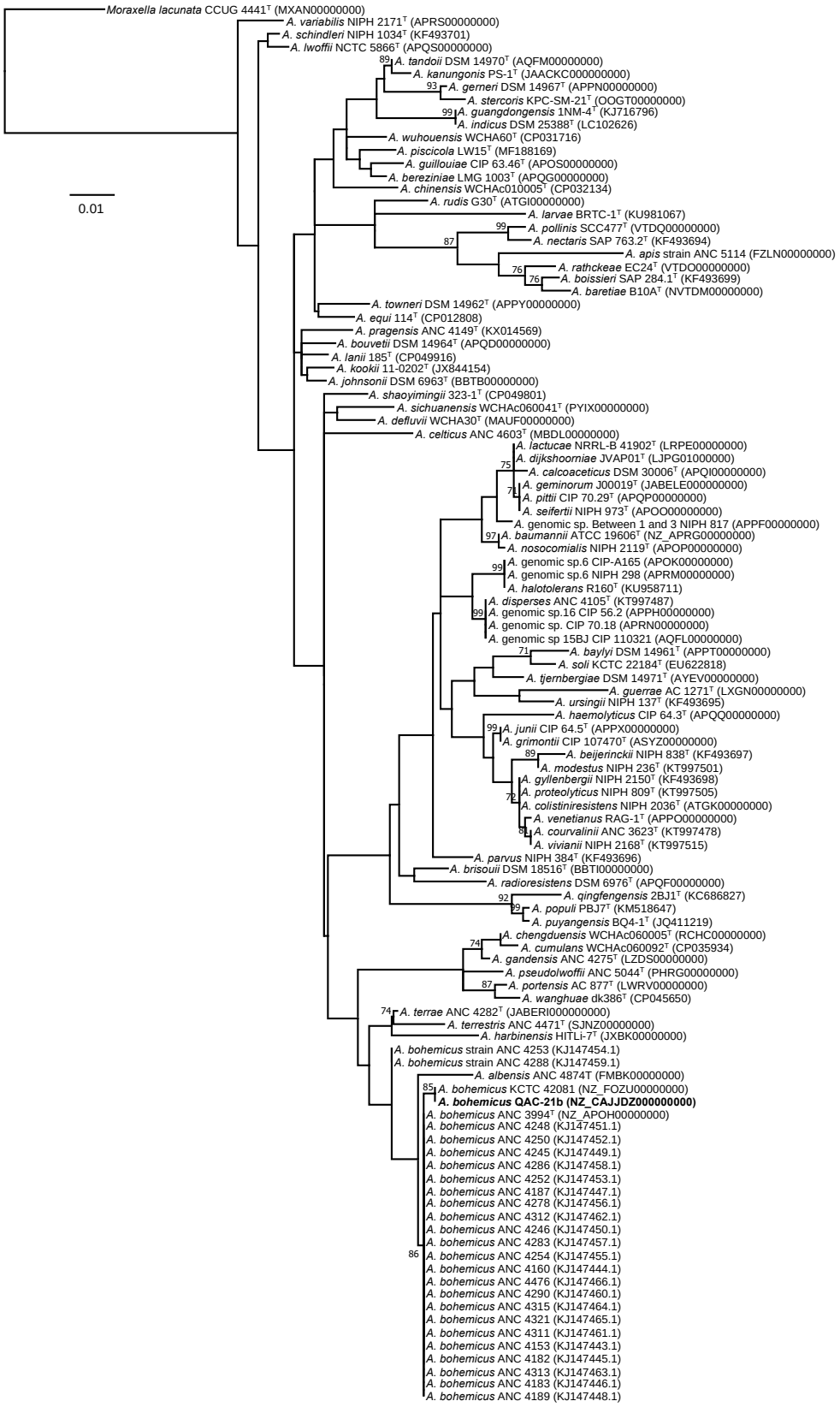

Figure S4. Phylogenetic tree based on partial amino acid sequence of *gyrB* showing the placement of QAC-21b into the species cluster of *A. bohemicus*. Phylogenetic

analysis was inferred by using the Maximum Likelihood method based on the JTT matrix-based model, applying a discrete Gamma distribution to model evolutionary rate differences among sites (+G), assuming some sites to be evolutionarily invariable (+I). Bootstrap values (≥70%) based on 100 re-samplings are shown above the branches. The analysis involved a total of 205 positions in the final dataset. Tree was calculated in MEGA7. Bar, rate of substitution per amino acid position.

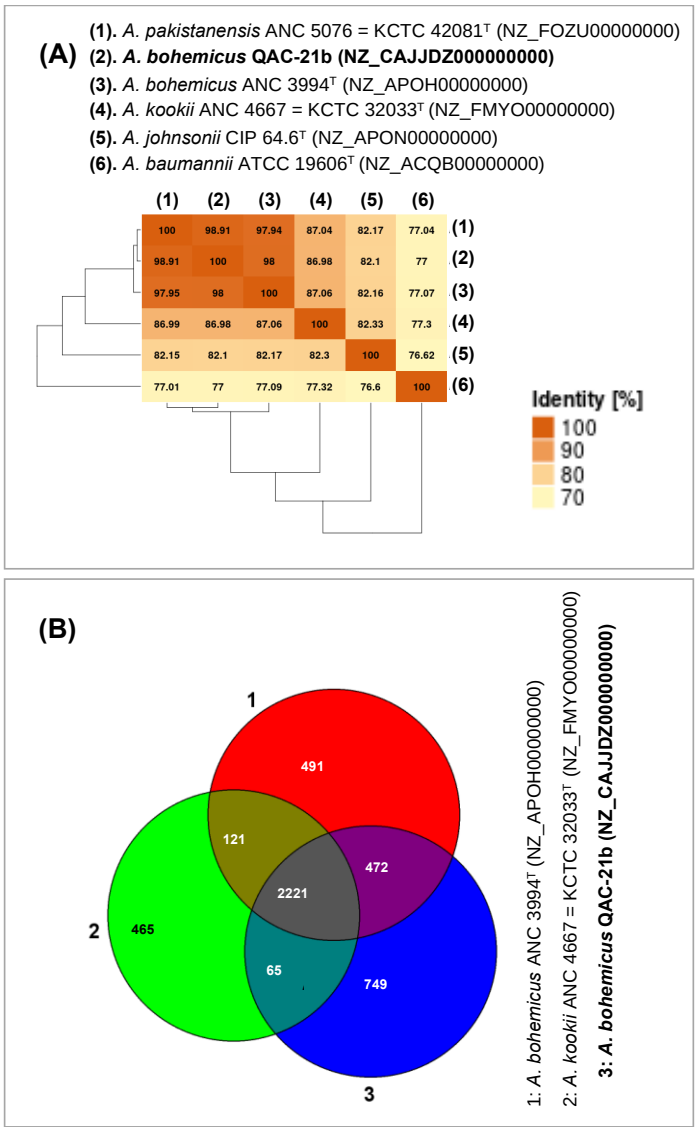

Figure S5. (A) Heat map of average nucleotide identity (ANI) values of strain QAC-21b compared to *A. bohemicus* strains ANC 3994^{T} and KCTC 42081 and other related *Acinetobacter* strains. (B) Venn diagram depicting unique and shared genes between QAC-21b and *A. bohemicus* ANC 3994^{T} and KCTC 42081.

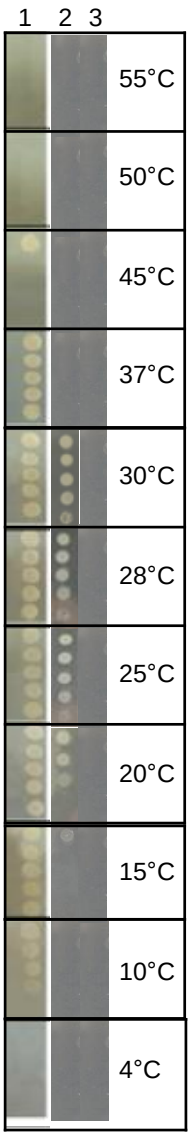

Figure S6. Temperature dependent growth of QAC-21b analyzed by the spot assay method according to Pulami et al. (2021). 1, *A. baumannii* ATCC 19606^T, 2, strain QAC-21b; 3, spotted 0.9% sodium chloride (NaCl) solution. Analysis was performed after 24h of incubation

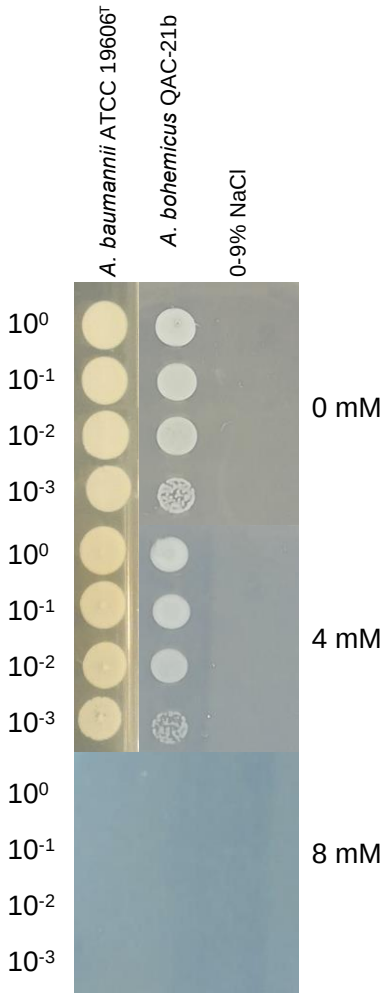

Figure S7. Copper tolerance of QAC-21b analyzed by the spot assay method according to Pulami et al. (2021). Copper tolerance was tested by pipetting 5 µL of serial dilution (10^0–10^{-3} dilution, turbidity adjusted to standard 0.5 McFarland) of overnight cultured isolates in Muller Hinton agar plates (ROTH) supplemented with 4, 8, 12, 16, 20, 24, 32 and 36 mM $CuSO_4$ $5H_2O$ (adjusted to pH 7.2) (MERCK). All plates were incubated at 25°C in the dark and checked for growth after 24h of incubation. The lowest copper concentration that completely inhibited growth was considered as the MIC value. The growth was completely suppressed in concentration of 8 mM $CuSO_4$ $5H_2O$ and above (data not shown).

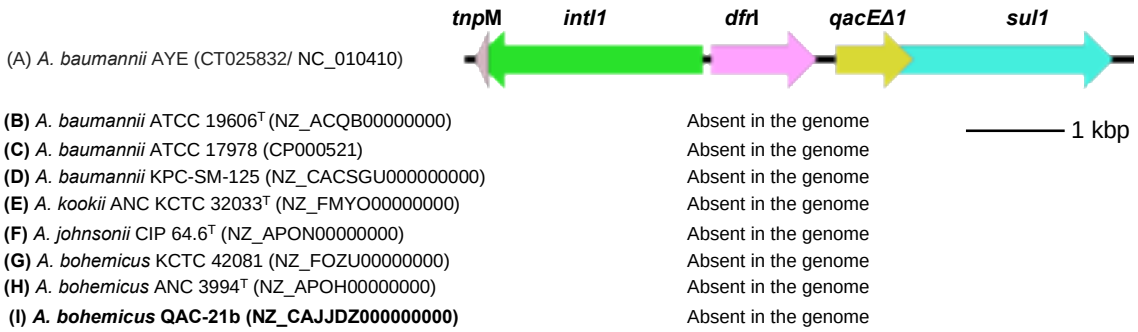

Figure S8. Comparison of operon of class 1 integron (*intI1*) with QAACs efflux pump QacEdelta1 (*qacEΔ1* gene) present in the chromosome of *Acinetobacter* strains. Figure and comparison were visualized using Easyfig v2.2.5 (Sullivan et al. 2011)

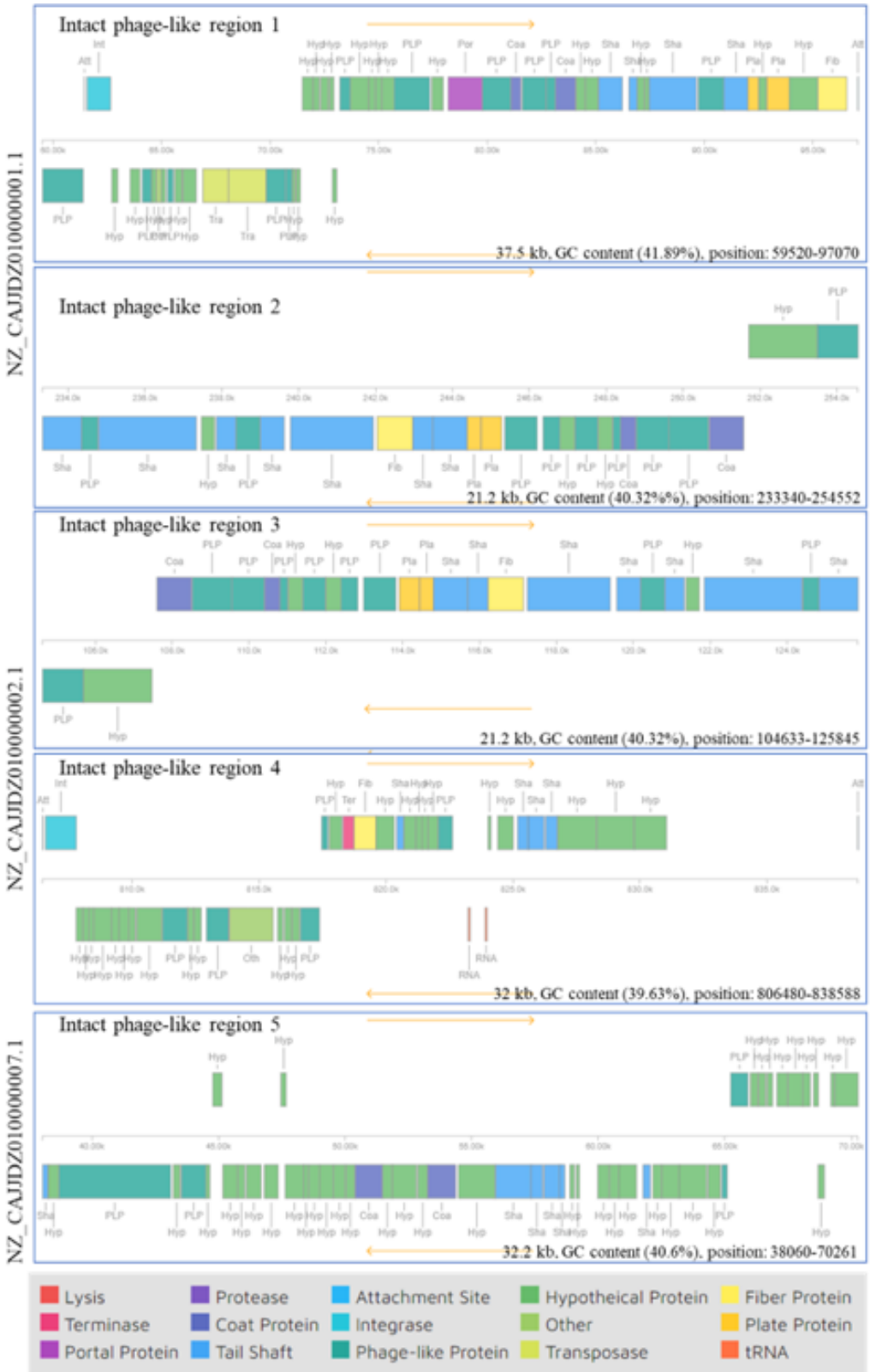

Figure S9. Details of intact phage-like regions in *A. bohemicus* QAC-21b showing the characteristics of phage-related genes

Figure S10. Details of intact phage-like regions in *A. bohemicus* ANC 3994^{T} showing the characteristics of phage-related genes.

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Annex

Chapter V, Supporting Information

Acinetobacter stercoris sp. nov. isolated from output source of a mesophilic german biogas plant with anaerobic operating conditions

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Antonie van Leeuwenhoek Journal of Microbiology (2021) 114:235–251

https://doi.org/10.1007/s10482-021-01517-7

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Supplementary tables

Table S1. Genome accession and assembly number of organisms (including type species and strain) used in this study.

Organism	GenBank Accession	GenBank assembly accession
Acinetobacter lwoffii NCTC 5866^T = CIP 64.10^T _= NIPH 512^T	APQS00000000	GCA_000369105.1
Acinetobacter baumannii ATCC 19606^T = CIP 70.34^T = JCM 6841^T	APRG00000000	GCA_000369385.1
Acinetobacter baylyi DSM 14961^T = CIP 107474^T	APPT00000000	GCA_000368685.1
Acinetobacter bereziniae LMG 1003^T = CIP 70.12^T	APQG00000000	GCA_000368925.1
Acinetobacter bohemicus ANC 3994^T	APOH00000000	GCA_000367925.1
Acinetobacter bouvetii DSM 14964^T = CIP 107468^T	APQD00000000	GCA_000368865.1
Acinetobacter brisouii CIP 110357	AYEU00000000	GCA_000488275.1
Acinetobacter calcoaceticus DSM 30006^T	APQI00000000	GCA_000368965.1
Acinetobacter equi 114^T	CP012808	GCA_001307195.1
Acinetobacter gandensis ANC 4275^T	LZDS00000000	GCA_001678755.1
Acinetobacter dijkshoorniae JVAP01^T	NZ_LJPG00000000	GCF_001595745.1
Acinetobacter gerneri DSM 14967^T = CIP 107464^T	APPN00000000	GCA_000368565.1
Acinetobacter guillouiae CIP 63.46^T	APOS00000000	GCA_000368145.1
Acinetobacter haemolyticus CIP 64.3^T	APQQ00000000	GCA_000369065.1
Acinetobacter harbinensis HITLi 7^T	JXBK00000000	GCA_000816495.1
Acinetobacter indicus CIP 110367^T = DSM 25388^T	BBSF00000000	GCA_000830155.1
Acinetobacter johnsonii CIP 64.6^T	APON00000000	GCA_000368045.1
Acinetobacter junii CIP 107470^T	APPS00000000	GCA_000368665.1
Acinetobacter junii CIP 64.5^T	APPX00000000	GCA_000368765.1
Acinetobacter lactucae NRRL B 41902^T	LRPE00000000	GCA_001605885.1
Acinetobacter nectaris CIP 110549	AYER00000000	GCA_000488215.1
Acinetobacter nosocomialis NIPH 2119^T	APOP00000000	GCA_000368085.1
Acinetobacter parvus DSM 16617^T	APOM00000000	GCA_000368025.1
Acinetobacter pittii ATCC 19004^T = CIP 70.29^T	APQP00000000	GCA_000369045.1
Acinetobacter radioresistens DSM 6976^T = CIP 103788^T	APQF00000000	GCA_000368905.1
Acinetobacter rudis DSM 24031^T	BBRX01000000	GCA_000829675.1
Acinetobacter schindleri CIP 107287	APPQ00000000	GCA_000368625.1
Acinetobacter seifertii NIPH 973^T	APOO00000000	GCA_000368065.1
Acinetobacter soli KCTC 22184^T	BBNM00000000	GCA_000760595.1
Acinetobacter tandoii DSM 14970^T = CIP 107469^T	AQFM00000000	GCA_000400735.1
Acinetobacter tjernbergiae DSM 14971^T = CIP 107465^T	AYEV00000000	GCA_000488175.1
Acinetobacter towneri DSM 14962^T = CIP 107472^T	APPY00000000	GCA_000368785.1
Acinetobacter ursingii DSM 16037^T = CIP 107286^T	APQA00000000	GCA_000368825.1
Acinetobacter venetianus RAG 1^T = CIP 110063^T	APPO00000000	GCA_000368585.1
Acinetobacter albensis ANC 4874^T	FMBK01000000	GCA_900095025.1
Acinetobacter defluvii WCHA30^T	MAUF00000000	GCA_001704615.2
Acinetobacter piscicola LW15^T	NIFO00000000	GCA_002233755.1
Acinetobacter populi PBJ7^T	NEXX00000000	GCA_002174125.1
Acinetobacter larvae BRTC-1^T	CP016895	GCA_001704115.1
Acinetobacter apis ANC 5114	FZLN00000000	GCA_900197575.1
"*Acinetobacter oleivorans* DR1 = KCTC 23045"	BCUK00000000	GCA_001591245.1
Moraxella lacunata NBRC 102154^T = CCUG 444^T	BCUK00000000	GCA_001591245.1

Table S2. Potential virulence factor related genes of strain KPC-SM-21^T. The genome of *A. baumannii* ATCC19606^T was used as reference. Analyses were performed in EDGAR 2.3 (Blom et al. 2016) and VFDB (virulence factor database; http://www.mgc.ac.cn/VFs/) which was used to indentify virulence related genes.

					Strain	
Gene/Locus/Operon	Protein	Predicted function	Role in pathogenesis	Reference for evidence	ATCC 19606T	KPC-SM-21T
Type 2 Secretion system					Locus tag	
gspN	GspN	Unknown function	In vivo survival	Johnson et al. 2016; Elhosseiny et al. 2016; Harding et al. 2016; Harding, Hennon and Feldman 2018	HMPREF0010_01960	KPC_2986
gspC	GspC	Inner-membrane platform protein			HMPREF0010_01959	KPC_2987
gspD	GspD	Outer-membrane secretion			HMPREF0010_01958	KPC_2988
gspL	GspL	Inner-membrane platform protein			HMPREF0010_00073	KPC_0719
gspM	GspM	Inner-membrane platform protein			HMPREF0010_00074	KPC_0720
gspE	GspE	Secretion ATPase			HMPREF0010_01637	KPC_1813
gspF	GspF	Inner-membrane platform protein			HMPREF0010_01870	KPC_2178
gspKJIH	GspKJIH	Minor pseudopilins			HMPREF0010_00793-00796	KPC_2625-2628
Type 6 secretion system					Locus tag	
tssL	TssL	membrane complex proteins	interspecies competition, host colonization	Weber et al. 2013; Carruthers et al. 2013; Wright et al. 2014; Jones et al. 2015; Repizo et al. 2015	HMPREF0010_01111	KPC_3150
tssK	TssK	baseplate components			HMPREF0010_01112	KPC_3149
tssA	TssA	Priming protein			HMPREF0010_01113	KPC_3148
tssH	TssH	ATPase ClpV			HMPREF0010_01114	KPC_3147
tagN	TagN	Structural component			HMPREF0010_01116	KPC_0890
tagF	TagF	Posttranslational repressor of T6SS			HMPREF0010_01117	KPC_0889
tssM	TssM	membrane complex proteins			HMPREF0010_01118	KPC_0888
tssG	TssG	baseplate components			HMPREF0010_01120	KPC_0886
tssF	TssF	baseplate components			HMPREF0010_01121	KPC_0885
tssE	TssE	baseplate components			HMPREF0010_01122	KPC_0884
hcp	Hcp	tubule protein			HMPREF0010_01123	KPC_0883
tssB	TssB	Sheath components			HMPREF0010_01125	KPC_0881

tssC	TssC	Sheath components			HMPREF0010_01124	KPC_0882
Others					Locus tag	
OmpA (Omp38)	Outer-membrane protein A	Outer membrance protein	Adherence, invasion, apoptosis, biofilm, persistence	Choi et al. 2005; 2008a, 2008b; Gaddy, Tomaras and Actis 2009; Lee et al. 2010; Smani et al. 2014; Wang et al. 2014	HMPREF0010_02782	KPC_1415
ptK	protein tyrosine kinase	Capsular polymerisation and assembly	Biofilm, tissue infection, serum growth	Russo et al. 2010	HMPREF0010_03290	KPC_2580
epsA	putative polysaccharide export outermembrane protein	Capsular polymerisation and assembly	Human serum resistance, in vivo survival	Russo et al. 2010	HMPREF0010_03288	KPC_2582
nfuA	Fe/S protein NfuA	iron-sulfur cluster biogenesis in iron deficiency	survival in vivo, hosts cell attack, persistence	Zimbler et al. 2012	HMPREF0010_01516	KPC_3562
plC1	Phospholipase C	catalysing the cleavage of phospholipids present in host cell membrance aiding cell lysis enzymatic catalysis		Camarena et al 2010	HMPREF0010_03297	KPC_3412
plD	Phospholipase D/cardiolipin synthase			Jacobs et al 2010	HMPREF0010_00607	KPC_2840
cpaA	metalloprotease CpaA	Inhibition of blood coagulation		Tilley et al. 2014; Kinsella et al. 2017	absent	KPC_0460

Table S3. Potential phage genes of strain KPC-SM-21^T. Contig sequences were examined for phage related genes using PHASTER (https://phaster.ca/; Zhou et al. 2011; Arndt et al. 2016).

No.	Contig Accession	Completeness	Score	Position	Most common phage	Phage accession
1.	NZ_OOGT01000171.1	Incomplete	10	124-5588	PHAGE_Acinet_YMC11/11/R3177	NC_041866
2.	NZ_OOGT01000144.1	Incomplete	20	58-9763	PHAGE_Acinet_AbP2	NC_041998
3.	NZ_OOGT01000254.1	Incomplete	10	797-4507	PHAGE_Burkho_Bcep176	NC_007497
4.	NZ_OOGT01000013.1	Incomplete	10	2434-8498	PHAGE_Acinet_Bphi_B1251	NC_019541
5.	NZ_OOGT01000018.1	Incomplete	30	5003-11252	PHAGE_Acidov_ACP17	NC_041997
6.	NZ_OOGT01000008.1	Intact	120	4305-38981	PHAGE_Acinet_YMC11/11/R3177	NC_041866

Supplementry figures

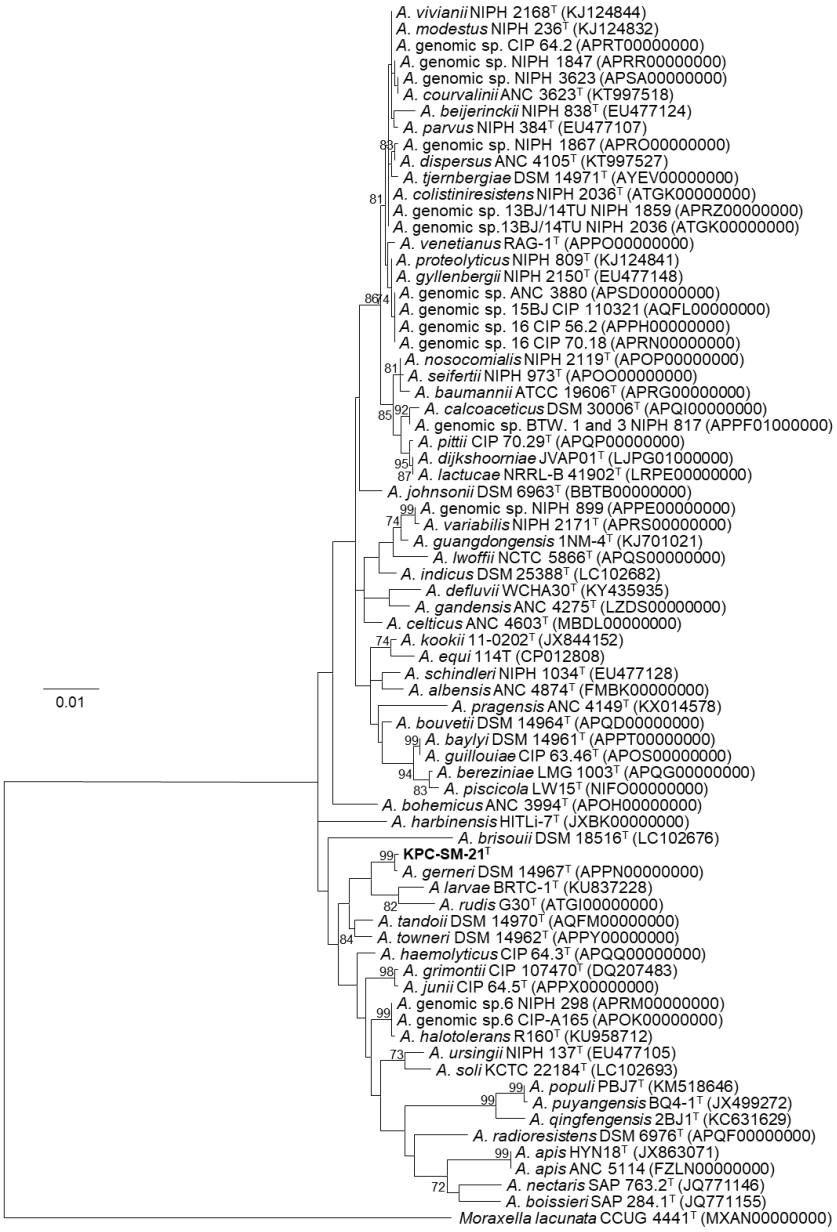

Figure S1. Maximum-likelihood (Felsenstein 1981) tree based on aminoacid sequences of concatenated variable zones of *rpoB* gene, showing the placement of KPC-SM-21^T within the genus *Acinetobacter*. Bootstrap values (>70%) based on 100 replicates are shown at the branch nodes. *Moraxella lacunata* NBRC 102154^T was used as outgroup. Bar, 0.01 substitutions per site.

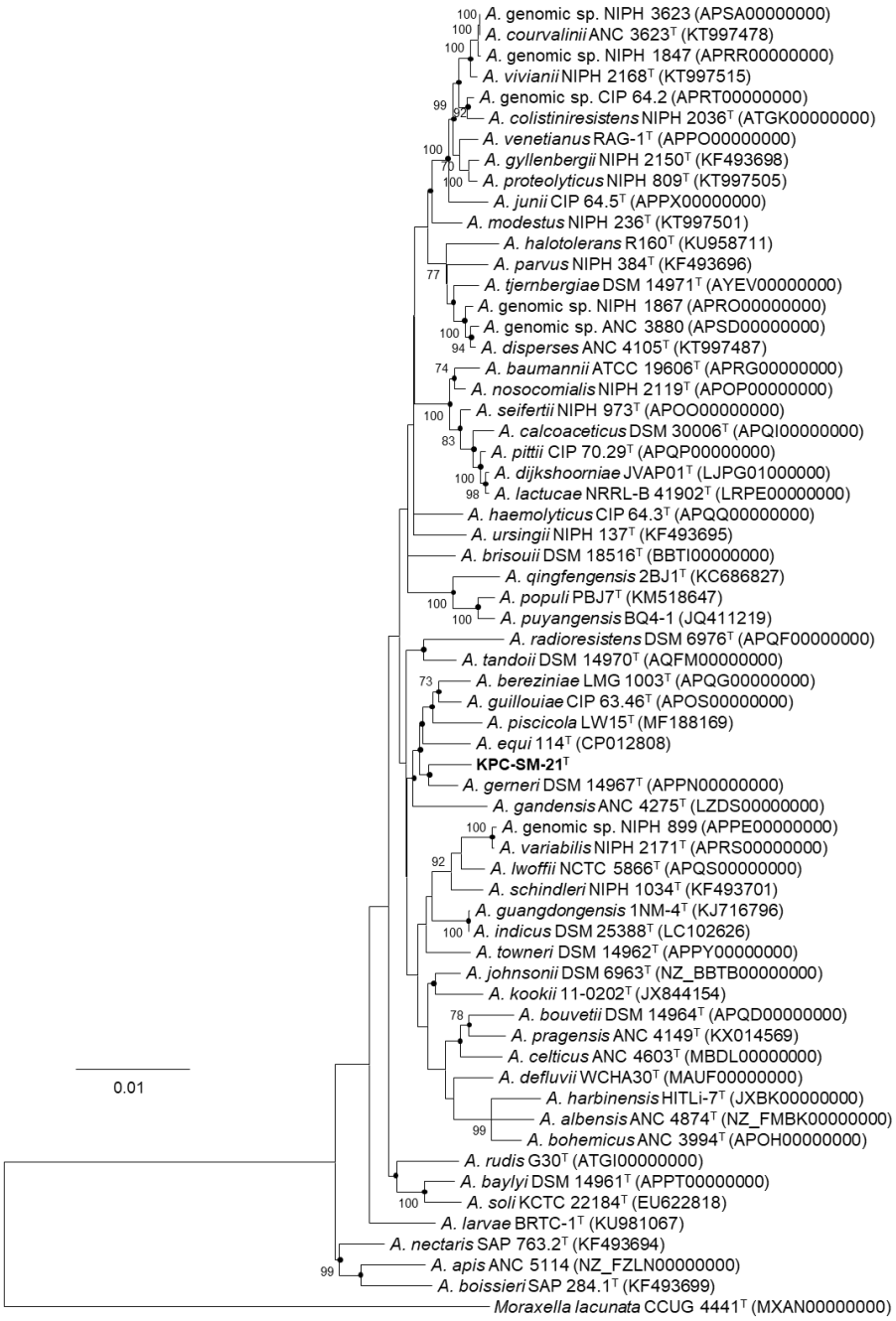

Figure S2. Maximum-likelihood tree based on short nucleotide sequence of *gyrB* gene (906nt), showing the placement of KPC-SM-21^T within the genus *Acinetobacter*. Bootstrap values (>70%; 100 replications) are shown. Filled circles indicate nodes that were also present in tree generated by neighbour-joining method. *Moraxella lacunata* NBRC 102154^T was used as outgroup. Bar, 0.01 substitutions per site.

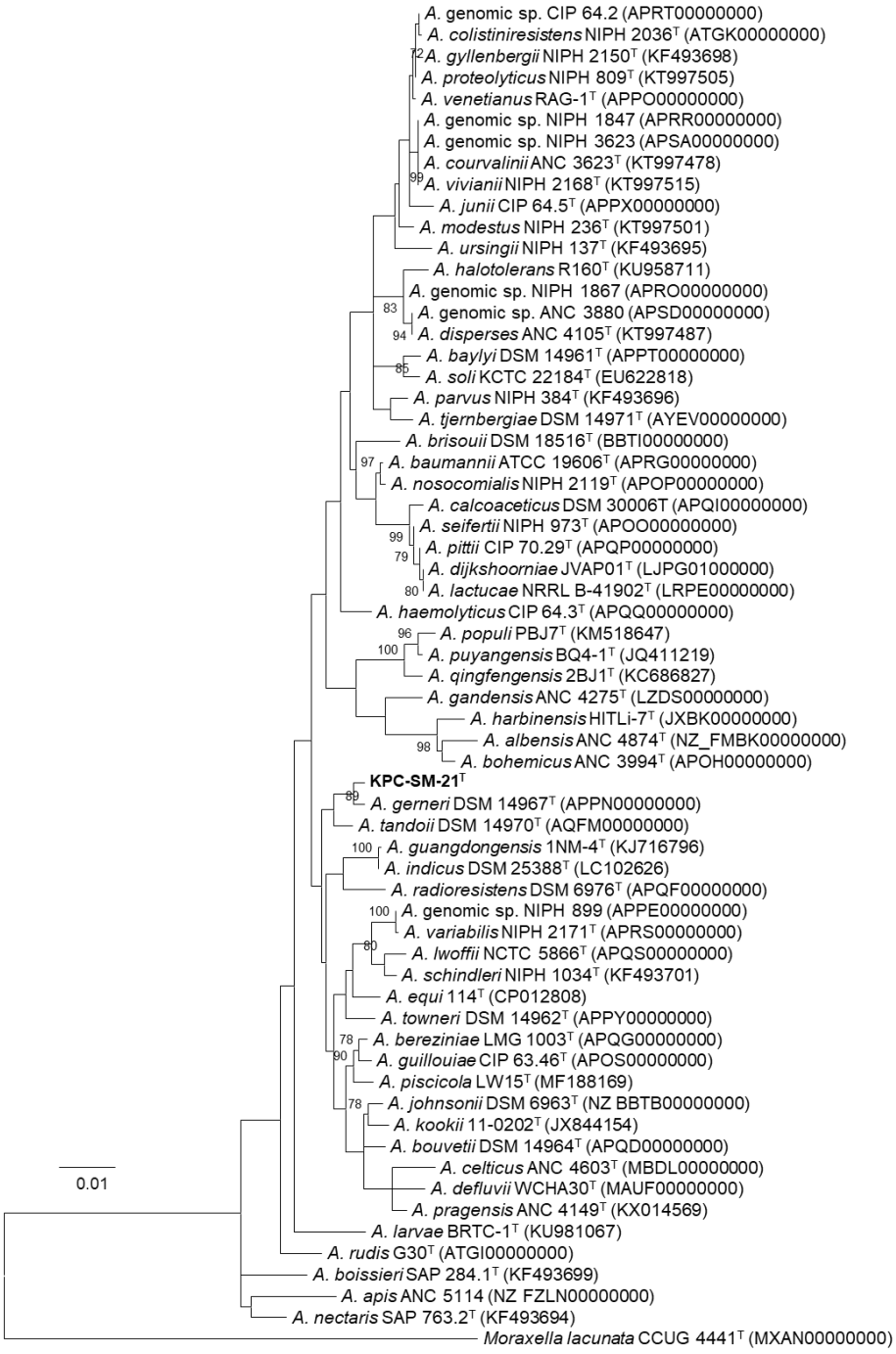

Figure S3. Maximum-likelihood tree based on aminoacid sequence of *gyrB* gene, showing the placement of KPC-SM-21^T within the genus *Acinetobacter*. Bootstrap values (>70%) based on 100 replicates are shown at the branch nodes. *Moraxella lacunata* NBRC 102154^T was used as outgroup. Bar, 0.01 substitutions per site.

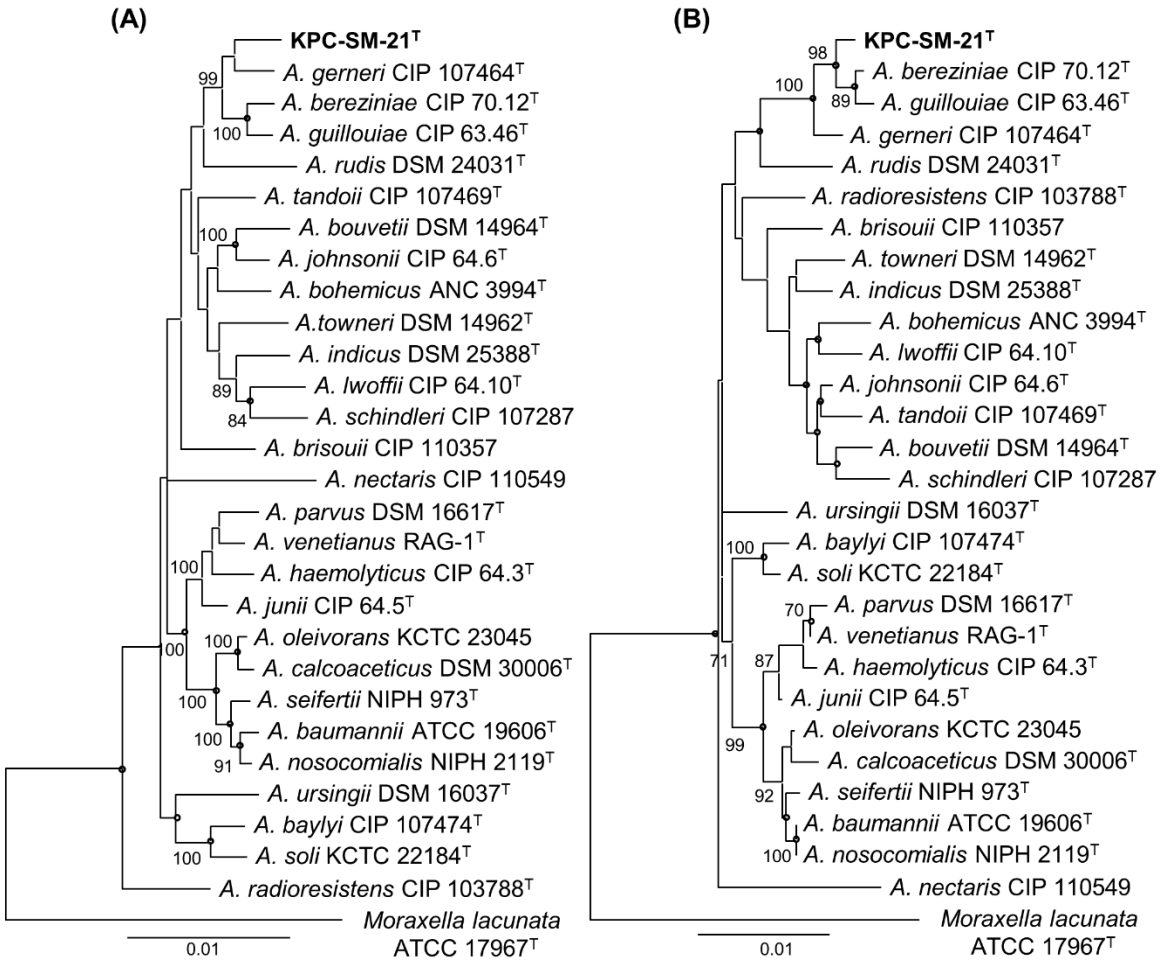

Figure S4. Phylogenetic placement of strain KPC-SM-21^T within the genus *Acinetobacter* based on a six gene MLSA. The phylogenetic tree was calculated with the ML method and is based on concatenated partial *pyrG-cpn60-gltA-recA-rplB-rpoB* nucleotide (A) and respective amino acid (B). Bootstrap values of ≥70% (100 replications) are shown. Phylogenetic analyses were based on a total of 2,331 nucleotide and 777 amino acid codon positions. Filled circles indicate nodes that were also present in trees generated by NJ and maximum parsimony methods. *Moraxella lacunata* ATCC 17967^T was used as outgroup. Bars, 0.01% sequence divergence. Locus tag number of *pyrG, cpn60, gltA, recA, rplB and rpoB* genes of strain KPC-SM-21^T are KPC_1268, KPC_2174, KPC_3048, KPC_3432, KPC_0262, and KPC_0582.

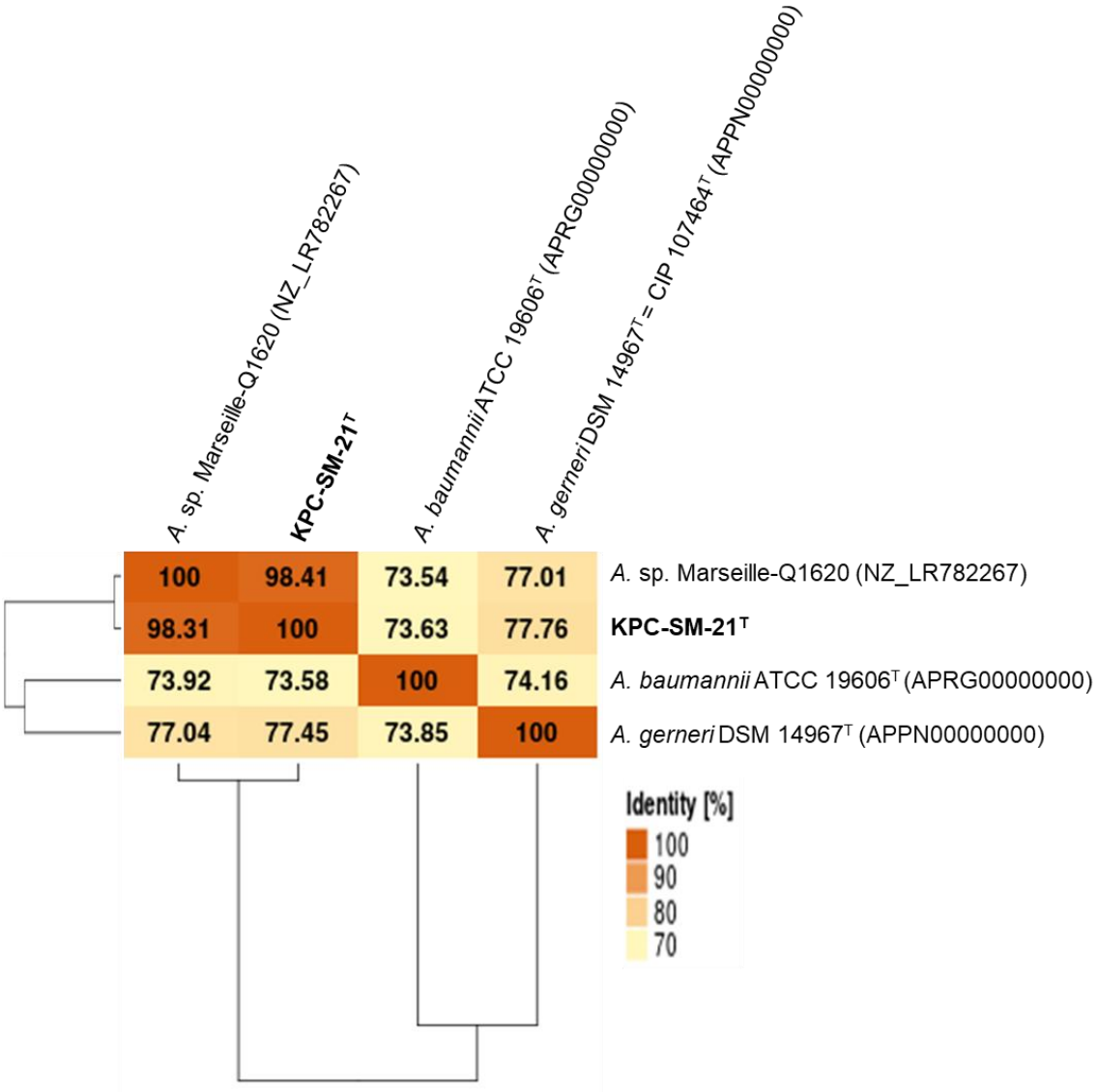

Figure S5. Clustered heat map of ANI values between the strain KPC-SM-21^T, *Acinetobacter* sp. Marseille-Q1620, *A. gerneri* DSM 14967^T and *A. baumannii* ATCC 19606^T. The analyses were done in EDGAR 2.3 (Blom et al. 2016).

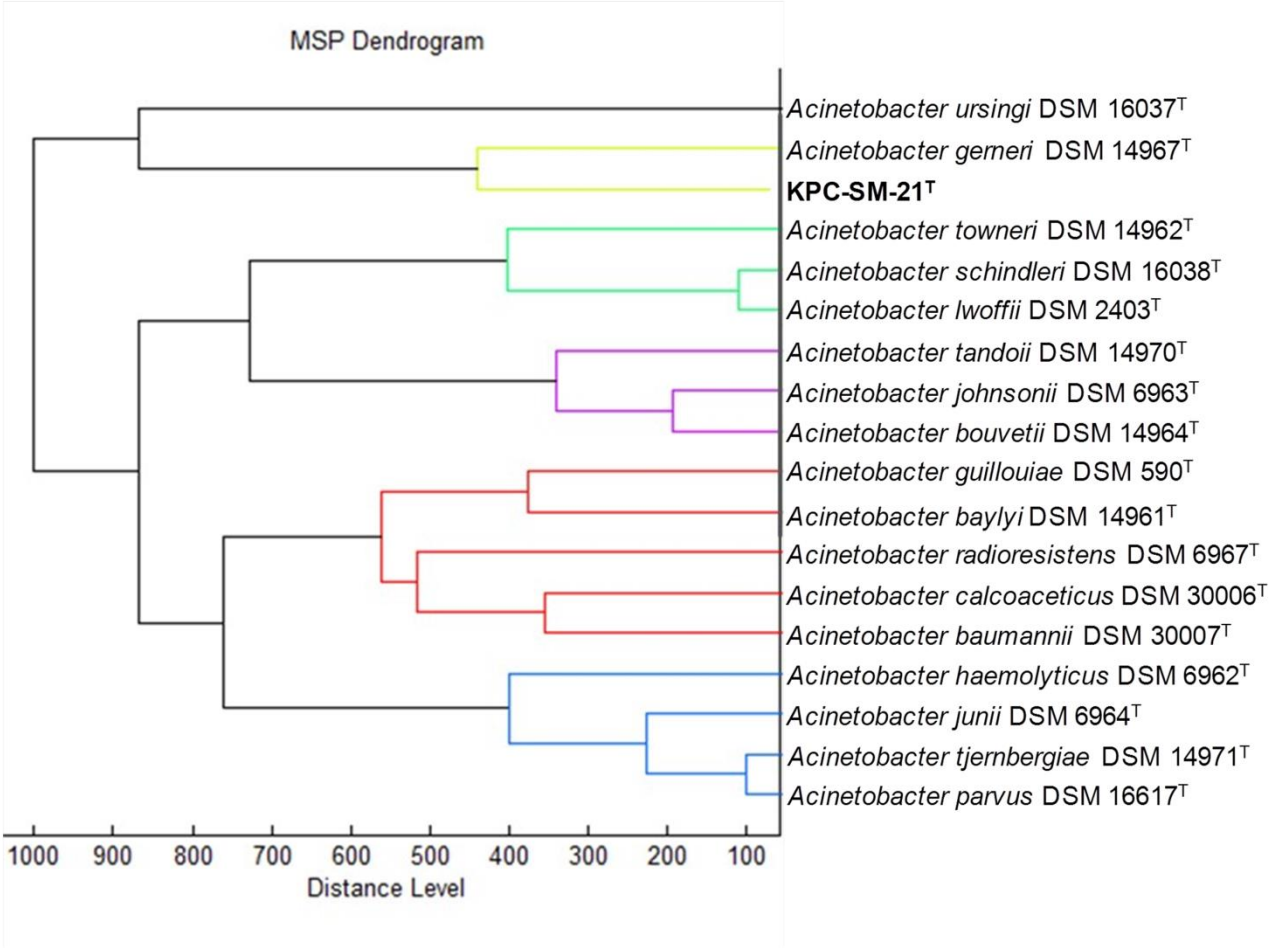

Figure S6. Biotyper (Bruker Daltonics) generated dendrogram based on whole cell mass spectrometry (MALDI-TOF-MS) of KPC-SM-21^T (shown in bold font) and other type species of the genus *Acinetobacter*.

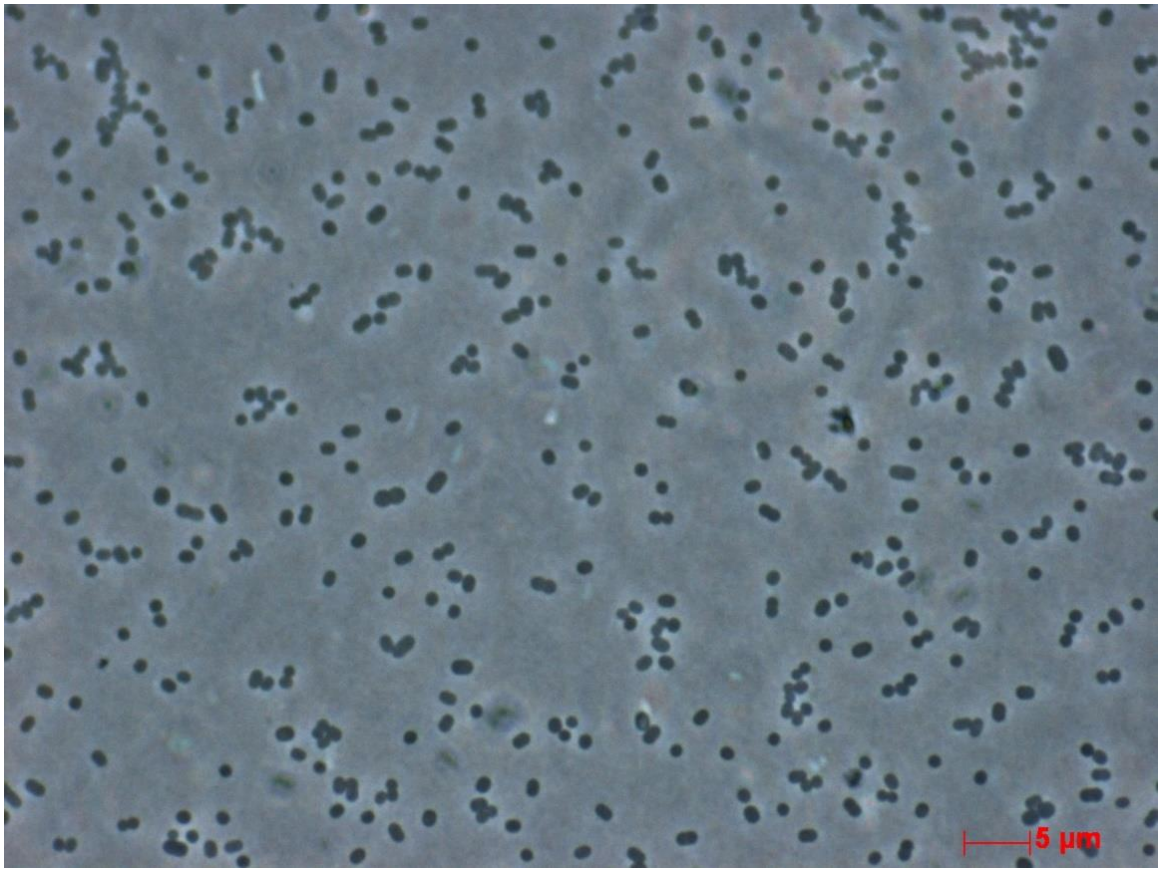

Figure S7. Microsopical picture of cellls of strain KPC-SM-21^{T}. Size: length (1.45µm) × width (1.2µm), Gram -negative, typically short, rod shaped (coccobacilli: intermediated between spherical and rod shape), non motile.

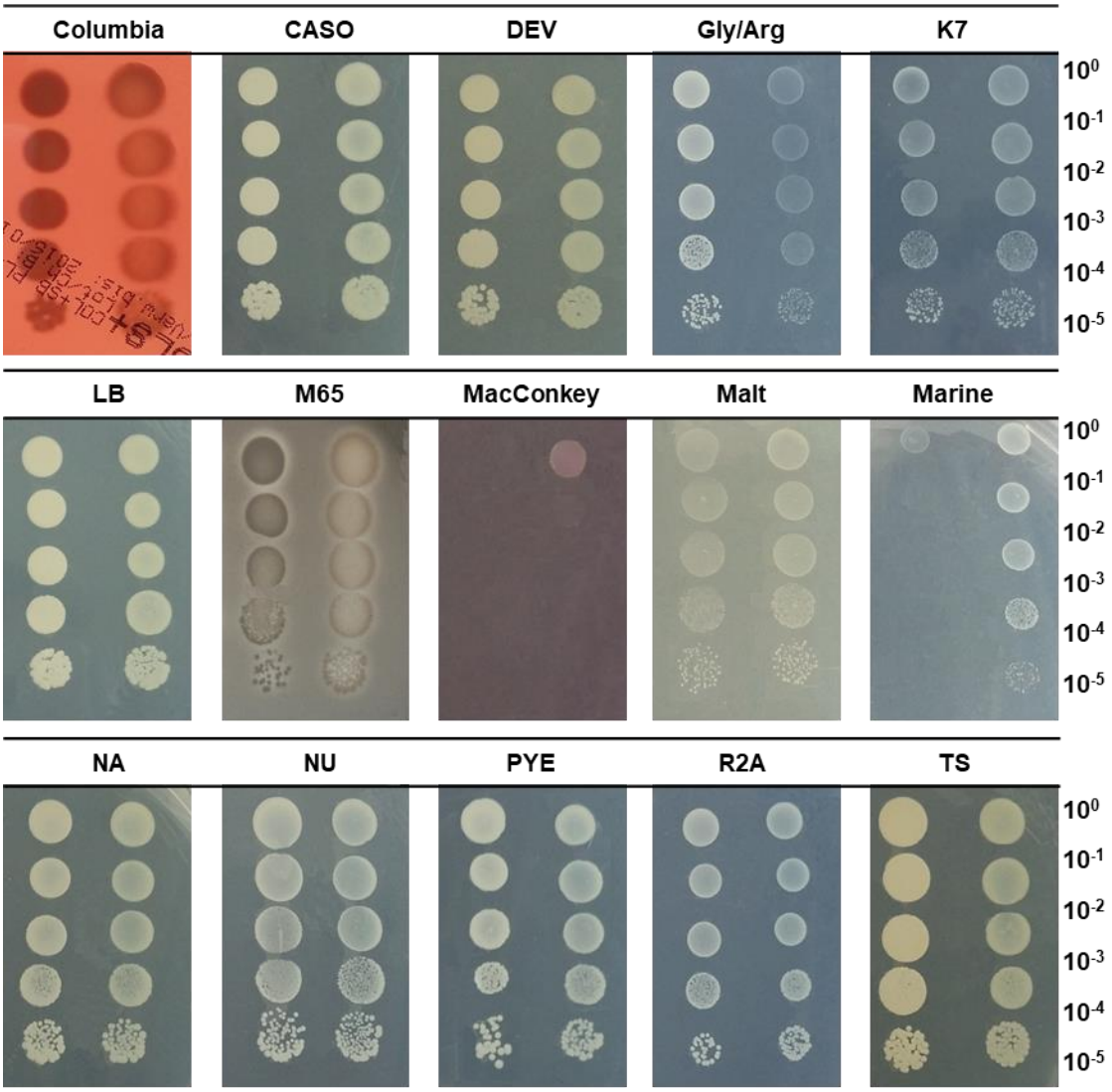

Figure S8. Spot assay test showing growth of strain KPC-SM-21^T (left) and *A. baumannii* ATCC 19606^T (right) on different growth media. Serial dilutions (10^0 to 10^{-5}) of cell suspensions (McFarland 0.5, suspended in autoclaved 0.9% NaCl solution) were spotted on each plate (5 µL per spot) and incubated at 28°C for 7 days. Growth of KPC-SM-21^T occurred in all media except marine agar (weak growth) and MacConkey (no growth). Images were taken after 7 days of incubation.

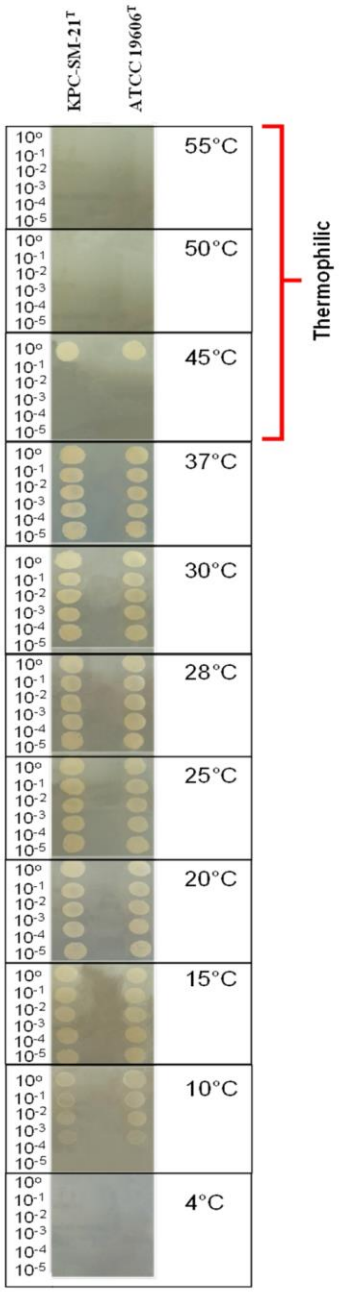

Figure S9. Spot assay of temperature dependent growth of strain KPC-SM-21^T and *A. baumannii* ATCC 19606^T following 24h of incubation.

Figure S10. Remnants of bacteriophage regions (Phage region 1) identified from querying the whole genome sequence of *Acinetobacter* sp. KPC-SM-21^{T} (Contig number: NZ_OOGT01000008.1) using PHASTER. The boxes are color coded with the legend provided below the figure to show their potential functions.

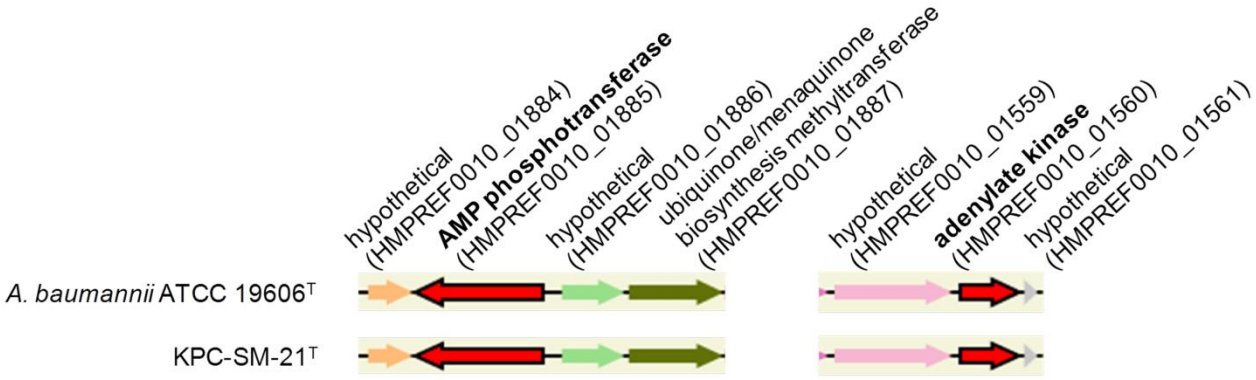

Figure S11. Distribution of genes encoding AMP phosphotransferase (PPK2) and adenylate kinase (adk) in the genome of *A. baumannii* ATCC 19606^T (ACQB00000000) and KPC-SM-21^T, respectively. The locus tag of AMP phosphotransferase and adenylate kinase are KPC_0195 and KPC_2791 for KPC-SM-21^T. Horizontal arrows indicate the direction and orientation of the gene.

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