The absence of the *ompA* and type VI secretion system in the successful lineage of *Acinetobacter baumannii* ST19

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Abstract

Acinetobacter baumannii is a critical, opportunistic pathogen due to its multi- to pandrug-resistance including resistance to last-resort antibiotics such as carbapenems. While *A. baumannii* can acquire environmental DNA through multiple mechanisms including natural competence, one of the most common is acquisition via plasmids by conjugation. One of the challenges for conjugation is an active Type VI Secretion System (T6SS), which kills nonkin bacteria and therefore inhibits the conjugation. The acquisition of resistance genes has helped the spread of resistant clones of *A. baumannii.* The most prominently disseminated clones belong to specific sequence types [(ST), Pasteur scheme], namely ST1, ST2, ST10, ST15, ST25, ST79 and ST85 with the ST2 being the most detected ST. While ST1 and ST2 were thoroughly studied, there are new emerging clones whose genetic background facilitating the rise is still unknown or poorly understood. One of the rising clones is *A. baumannii* ST19, recently reported to be frequently occurring in Georgia and Ukraine. In this study, we have whole-genome sequenced three isolates of *A. baumannii* ST19 which were then assessed for biofilm production and virulence in the *Galleria mellonella in vivo* infection model. Further 156 *A. baumannii* ST19 genomes were obtained from public repositories. In total, 157/159 genomes revealed loss of *ompA* and T6SS locus, which was in 56/157 genomes (35,7%) replaced by ΔTn*9* carrying chloramphenicol and ΔTn*10* carrying tetracycline resistance genes and formaldehyde and chlorite resistance genes. Hindered T6SS-loss-mediated contact-killing might promote the acquisition of genetic material supporting the success of the *A. baumannii* ST19 lineage.

Importance

Acinetobacter baumannii is a nosocomial pathogen with immense potential to become multidrug- to pan-drug-resistant, driven in part to its capacity to acquire DNA from the environment through conjugation and natural competence. Conjugation might however be hindered by an active type VI secretion system (T6SS), which kills nonkin bacteria. In *A. baumannii* ST19, the absence of the T6SS enables the co-existence and conjugation of nonkin bacteria. This, together with the reduced metabolic burden of lacking T6SS and further acquisition of antibiotic and non-antibiotic resistance traits, might enable the emergence of a successful, resistant, and potentially high-risk *A. baumannii* lineage ST19.

Introduction

Acinetobacter baumannii is an emerging opportunistic nosocomial pathogen that causes a variety of infections including urinary tract infections, wound infections, meningitis, ventilator-associated pneumonia, blood-stream infection, and endocarditis (1). *A. baumannii* is a member of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) (2), and due to its frequent resistance to last-line antibiotics such as carbapenems, it has been designated as a critical priority in the list of pathogens of importance for studying and developing new antibiotics by the World Health Organization's list (3, 4).

Besides conventional antibiotics, *A. baumannii* can withstand challenging environment conditions such as oxidative stress, prolonged periods of desiccation, human serum but also disinfectants (5).

The ability of *A. baumannii* to acquire exogenous DNA via natural competence (6) and conjugation (7) contributes to high plasticity of its genome (6). The horizontal gene transfer plays significant role in the evolution of *A. baumannii* lineages, resulting in number of highly successful clonal lineages such as ST1, ST2, ST10, ST15, ST25, ST79 and ST85 (Pasteur scheme) (8, 9). While ST1 and ST2 are responsible for the majority of infections caused by extensively-drug resistant *A. baumannii*, some clones are specific to certain geographical locations (9) , such as ST25 (Europe, United Arab Emirates, South America, Asia) (10) , ST32 (South Korea, Europe, the USA, Canada and the Middle East) (11) or ST78 (Italy, Ukraine, USA) (12).

While the horizontal gene transfer, especially plasmid transfer via conjugation, can shape entire lineages and their success (13) an active Type VI secretion system (T6SS) poses a unique challenge to conjugative plasmids, as *Acinetobacter* plasmid donors and recipients may kill each other (14). The T6SS machinery, including outer membrane protein *ompA*, is usually encoded in a single chromosomal locus (15). The T6SS of *A. baumannii* has been shown to kill *E. coli*, *K. pneumoniae* and *P. aeruginosa*, while different strains of *A. baumannii* are also able to kill each other (15, 16). While the role of *ompA* was demonstrated to influence the virulence and biofilm formation of *A. baumannii* (17) and was as well proposed as a possible therapeutic and vaccine target (18) the role of T6SS in *Galleria mellonella* model of virulence is believed to be indirect and strain dependent (19). Similarly, the presence T6SS in *A. baumannii* was also linked to higher biofilm-forming activity and better survival in the presence of normal human serum (20). Notably, the OmpA is rather required for the formation of thick biofilms on polystyrene, than essential for biofilm

development, (21) and implicated in inflammation, tissue destruction in a murine model of infection (22).

In this study, we have characterized a rising lineage of *A. baumannii* ST19, of which occurrence is increasing regionally (Ukraine, Georgia) (23) in its ability to form biofilm on abiotic surfaces and its virulence potential in *G. mellonella in vivo* infection model. Furthermore, we have performed comparative genomic analysis of the global population of *A. baumannii* ST19 in order to decipher genomic traits that make this lineage emergingly successful and to assess its threat in becoming world-wide disseminated clone.

Results

***A. baumannii* ST19 strains and genomes**

A concerning report on the increasing occurrence of *Acinetobacter baumannii* ST19 encoding carbapenemases was recently published (23), raising questions about factors contributing the success of this lineage. Three isolates of *A. baumannii* ST19 were obtained from two different patients (patient 1 - wound, rectal swab; patient 2 - rectal swab) and long-read whole-genome sequenced and *de novo* assembled.

A further 156 genomes (including 97 from Luo et al. (23)) of *A. baumannii* ST19 were retrieved from public repositories (GenBank and BIGSdb). *A. baumannii* ST19 has been occurring with increased frequency (Figure 1), suggesting it is successfully adapted to the hospital environment, the host or both.

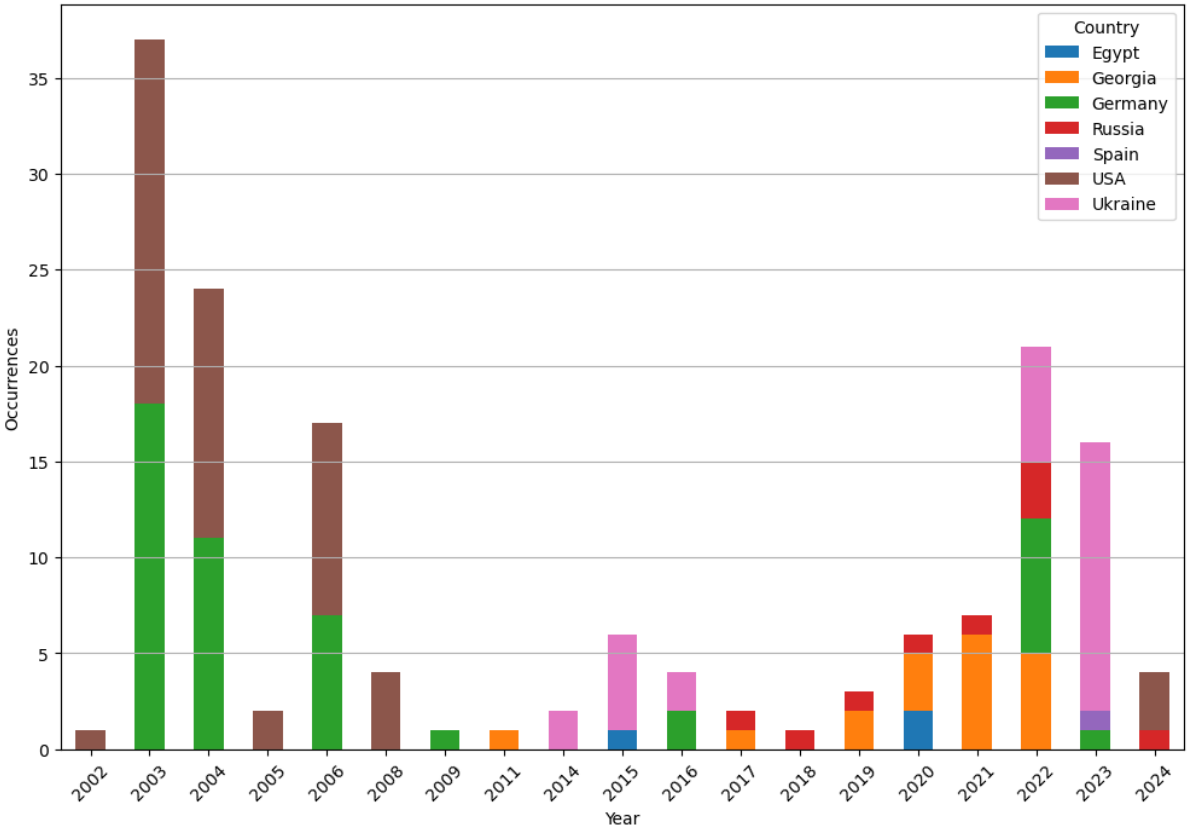

Figure 1: Temporal and spatial occurrence of *A. baumannii* ST19

The examined genomes carried a variety of virulence and resistance genes (Figure 2), including resistance to last-resort antibiotics such as carbapenems. One-third (n=53) out of 159 genomes described in this study encoded at least one carbapenemase gene (bla_{OXA-23}, bla_{OXA-72}, bla_{NDM-1} or bla_{NDM-5}) with 6 encoding two and 9 encoding three carbapenemases. Furthermore, a broad variety of plasmid replicons was detected (Figure 2), suggesting easy acquisition of genetic material.

Figure 2: BOTTOM

Figure 2: Phylogenetic analysis of 3 isolates and further 156 of *A. baumannii* ST19 genomes, depicting year of isolation, country, capsule locus type (KL), outer core lipooligosaccharide type (OCL), and presence of GI*sul2* genetic island, chlorite

dismutase, resistance, T6SS, formaldehyde, plasmid replicons, resistance, and virulence genes, respectively.

Genomic analysis suggests that the possible underlying reason for its success to be the acquisition of multiple partial transposons, including ΔTn*9* (24) carrying chloramphenicol resistance gene and ΔTn*10* carrying the tetracycline resistance gene. Interestingly, 61/159 (38,4 %) genomes of *A. baumannii* ST19 carried chlorite dismutase and formaldehyde resistance (*frmRAB*) genes, with 98,4% AA identity to *Massilia glaciei* and 96,7% to *Ideonella dechloratans*, where it combats oxidative stress (25). To our knowledge, this is the first time chlorite dismutase has been reported in the genome of *A. baumannii*.

Furthermore, these transposons disrupted and replaced the locus encoding *ompA* and the T6SS, impairing persistence as the examined isolates were weak biofilm producers (Figure 3). The entire region of T6SS and *ompA* deletion exhibits extensive genetic rearrangements, as ΔTn*9* and ΔTn*10* were acquired and disrupted by IS*26* and IS*4321,* and IS*26*, respectively.

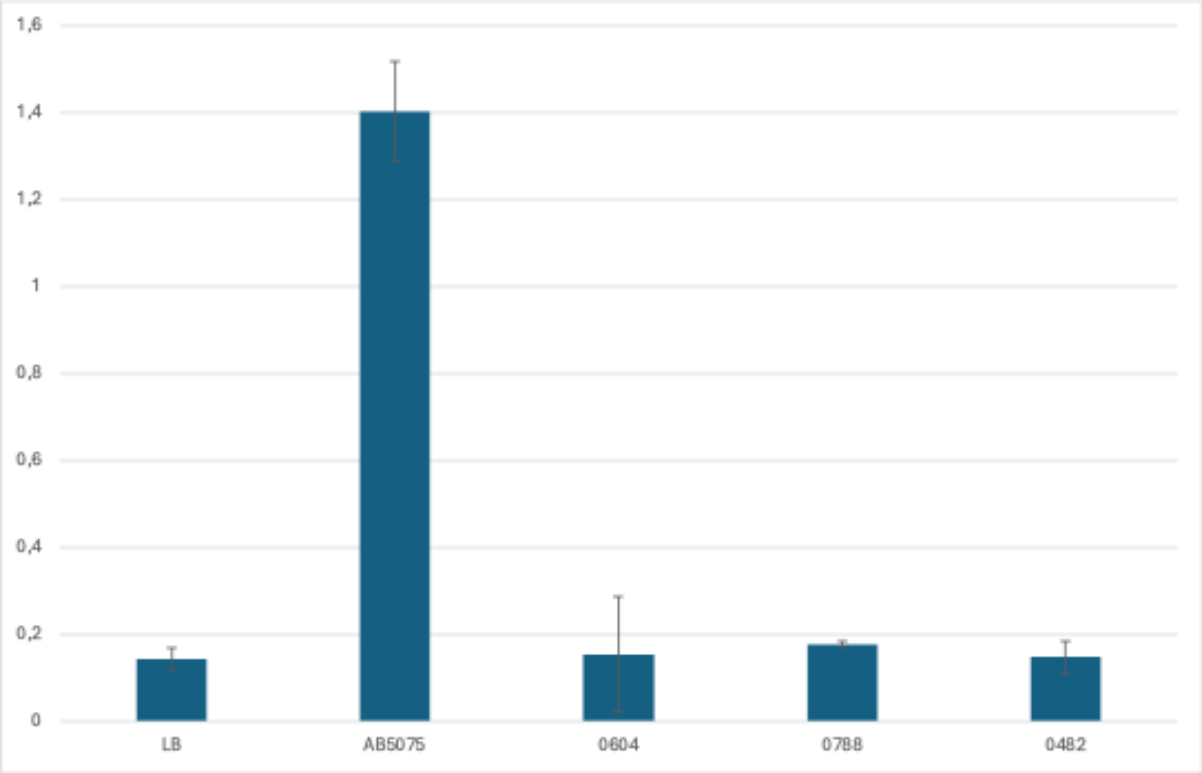

Figure 3: Crystal violet quantification of biofilm production in three ST19 strains of *A. baumannii*, AB5075 and non-inoculated LB medium as a control.

However, the virulence in the *G. mellonella* infection model varied (Figure 4), supporting the observation that T6SS is not directly involved in virulence in *G. mellonella* (19). The absence of T6SS, enabling conjugal acquisition of genetic material (14) such as antibiotic, formaldehyde and chlorite resistance, could explain their successful rise and frequent occurrence.

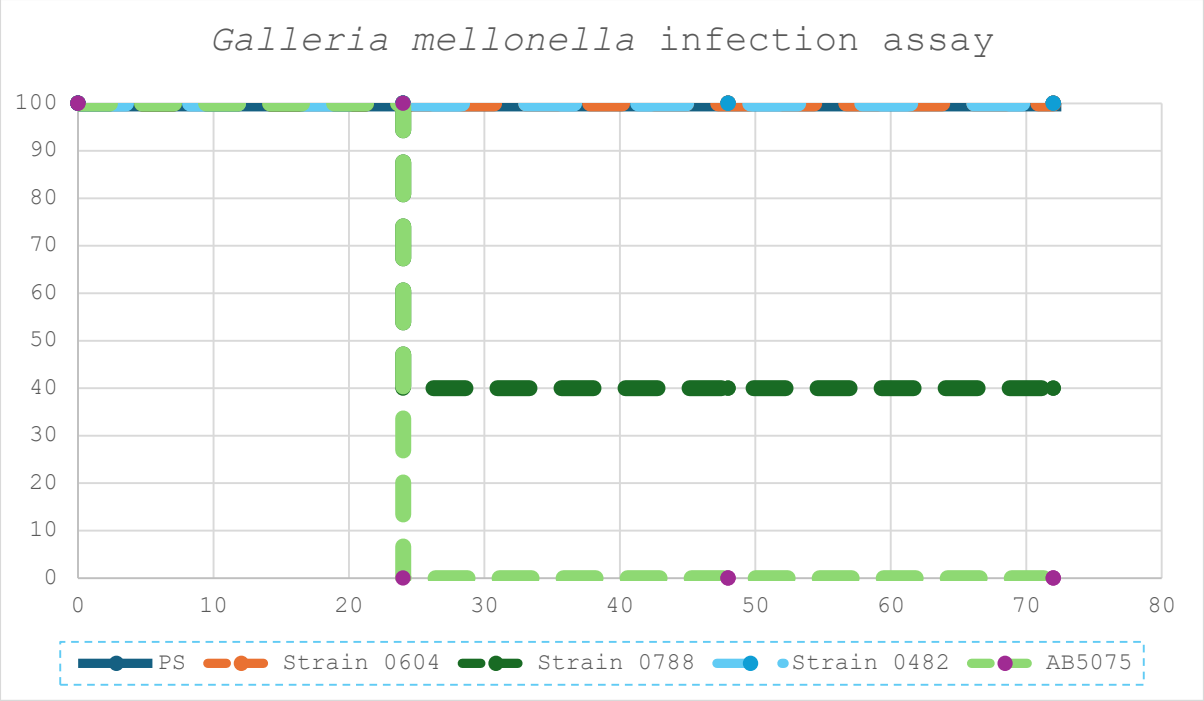

Figure 4: *In vivo* virulence in *G. mellonella* of three ST19 *A. baumannii* strains and AB5075 showing lower virulence potential of *A. baumannii* ST19 compared to AB5075 at infection dose of 2 × 10^7 CFU/mL. The x axis shows percentage of survival and the y axis the elapsed time in hours.

Genetic variability of the *A. baumannii* ST19 lineage

The whole genome sequences were subjected to genotyping [sequence type, capsule locus (KL) type, outer core lipooligosaccharide locus (OCL) type, antimicrobial resistance, and virulence genes]. A phylogenetic analysis was performed on these isolates, including genomes of the same ST from public repositories (GenBank and BIGSdb) (Figure 2).

The vast majority (157/159) of *A. baumannii* ST19 harbored GI*sul2,* a chromosomally carried Genomic Island encoding sulphonamide resistance determinant (*sul2*) along with putative arsenic resistance genes (26). We have detected a range of 0 to 6 plasmid replicons (27) in the genomes of *A. baumannii*

ST19, with rP-T1 replicon being the most prevalent (n=122/159), followed by r3-T23 (n=46/159), r3-T12 and r1-T8 (n=44/159 both).

Interestingly, 18 isolates did not carry any detectable plasmid replicon raising a question whether there is another mechanism limiting acquisition of plasmids or whether they have not encountered conjugative plasmid yet. However, the *Acinetobacter* typing schemes are still being developed (27), possibly undetected (or below the detection threshold) plasmids must be considered as well.

While various ST (Pasteur scheme) such as ST2 or ST1 often belong to various KL types (6), the ST19 lineage exhibited limited variability with 69/159 genomes being KL91, 66/159 KL15, followed by KL1 (13/159), KL147 (8/159), KL17 (2/159) and KL40 (1/159). Interestingly, 53/55 OCL1 encoded KL15 (2/55 OCL1 were KL17), the most predominant OCL type, OCL5 (n=87) was variable and encompassed KL91, KL1, KL147 and KL15. Furthermore, all of the genomes of KL15/OCL1 formed one large, genetically related cluster (Figure 2).

The capsule abundance was quantified in a gradient of silica. While the relative abundance was same for the tested *A. baumannii* ST19 isolates, it was lower amount of capsular polysaccharide than in AB5075 (Figure 5). While the capsulation level was same for all three isolates, the virulence phenotype was not identical (Figure 4), supporting previous observations of high capsulation not correlating with higher virulence potential (6).

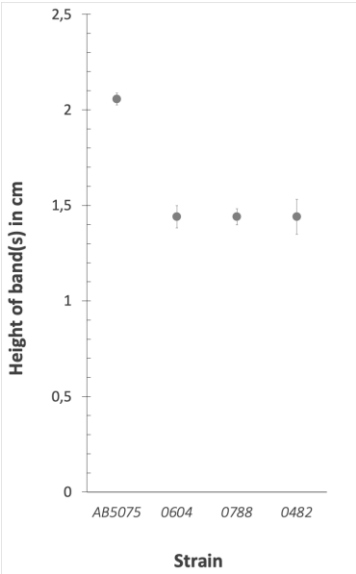

Figure 5: The quantification of the capsular polysaccharide production in the gradient of colloidal silica (Ludox LS) showing relatively high capsulation of all three tested *A. baumannii* ST19 strains. The AB5075 strain was tested as a reference.

Evaluating the T6SS locus in the ST19 lineage of *A. baumannii* we identified variability in genetic origin of the deletion of these genes. Of 61/159 genomes carrying chlorite dismutase and formaldehyde resistance genes in the locus of T6SS and *ompA*, 55/61 harbored ΔTn*10* carrying the tetracycline and ΔTn*9* carrying chloramphenicol resistance genes (Figure 6). Five formaldehyde resistance and chlorite dismutase-encoding genomes (5/61) did not harbor ΔTn*10* carrying the tetracycline resistance and 1/61 did not harbor ΔTn*9* carrying chloramphenicol resistance genes. However, mapping on the reference genome (0604 - SAMN46706488) confirmed, that in all these genomes (61/159) these genetic rearrangements displaced locus encoding T6SS and the *ompA* gene.

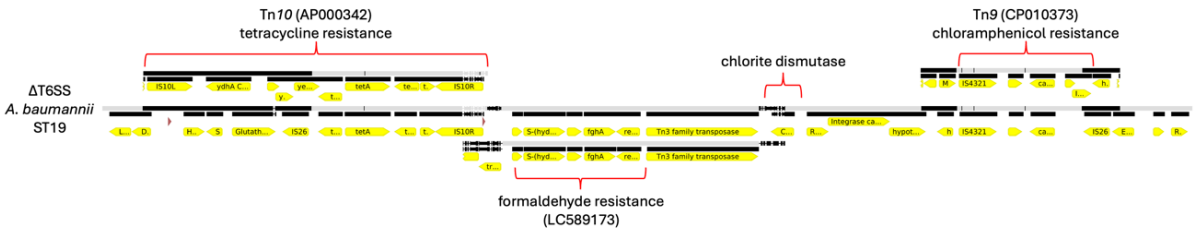

Figure 6: Former T6SS and *ompA* locus of *A. baumannii* ST19 (0788 - SAMN46706486) as a representative genome showing deletion mediated by ΔTn*10*, ΔTn*9*, and formaldehyde resistance locus.

Interestingly, 2/159 genomes of *A. baumannii* ST19 (GCA_042643835.1 and GCA_040868255.1) still encoded T6SS and *ompA* locus. However, compared to AB5075-VUB (accession nr. CP070362), a number of SNPs (99% pairwise identity over 19417 bp) was detected in these two genomes, while the broader genetic neighbourhood only sporadically exhibited SNPs. The genomes GCA_042643835.1 and GCA_040868255.1 belonged to KL40/OCL2 and KL17/OCL1, respectively, which are rare for this lineage (a sole KL40 and one of two KL17, respectively). While none of the SNPs were non-sense, the influence of missense or synonymous (e.g., via tRNA) on the functionality of T6SS remains to be explored.

Discussion

Acinetobacter has the core components of the T6SS conserved and found in a single chromosomal locus (15). The *Acinetobacter* strains use type VI secretion system (T6SS) to kill nonkin bacteria such as *E. coli*, *K. pneumoniae* and *P. aeruginosa* (16, 28). The active T6SS is also a challenge to conjugation and horizontal gene transmission (15). The absence of T6SS could therefore allow co-existence of nonkin bacterial species and strains. We hypothesize that this might and explain original

acquisition of genetic material from these species and possibly evolution of the *A. baumannii* ST19 lineage.

While the Tn*10* (29) was described, the best match of its nucleotide sequence was detected with *S. enterica* serovar Typhi and *S. flexneri*. The ΔTn*10* observed in this study has the highest similarity to regions from *Enterobacter hormaechei* IncHI2 palsmids (>99% nucleotide identity and 97% query coverage). Furthermore, the ΔTn*9* (30) examined in this study was BLASTn-detected (with >99% nucleotide identity and 100% query coverage) in *Klebsiella pneumoniae* IncF plasmids.

Although *adhC* genes facilitating resistance to formaldehyde which are native to *Acinetobacter* have been reported (31), we have identified locus, likely originating from *Enterobacter hormaechei* IncHI2 plasmid, or *Klebsiella pneumoniae* (or *quasipneumoniae*) IncF plasmid (95% nucleotide identity and 100% coverage). However, the origin of the chlorite dismutase gene remains undeciphered, while the BLASTn of here observed chlorite dismutase results in identical hits (>96% nucleotide identity and 98% query coverage) in various species, such as *Pseudomonas aeruginosa* (chromosome), *S. enterica* (IncHI2 plasmid), *K. pneumoniae* (IncF plasmid), *E. hormaechei* (chromosome), but also *Vibrio rumoiensis* (plasmid) *Raoultella ornithinolytica* (IncF plasmid) *Leclercia adecarboxylata* (IncHI2 plasmid).

Interestingly, the virulence in *G. mellonella* in this study varied, supporting previous observations that T6SS is not directly implicated in pathogenicity, and the virulence in *G. mellonella* might be indirect (19). As isolates with T6SS disrupted by IS*Aba1* were observed (32) it has been suggested that T6SS is not essential to cause the disease.

The OmpA is an outer membrane protein (33), encoded in the T6SS locus and is known to be involved in the formation of a translocation pore (34). The OmpA has been extensively studied for its role in *Acinetobacter* virulence (35). Furthermore, the OmpA plays a role in the development of robust biofilms on plastic, and A549 human alveolar epithelial cells (18, 21). This supports the observation of low biofilm forming ability of strains examined in this study. Furthermore, the loss of the *ompA* gene in the *A. baumannii* ST19 may have further consequences as it might render one of the most prominent vaccines targets ineffective (18, 36).

Conclusion

A. baumannii ST19 has been occurring with increased frequency, suggesting it has successfully adapted to the hospital environment. The loss of T6SS in the (nearly) entire lineage of *A. baumannii* ST19 points towards a beneficial evolutionary step, a fitness trade-off towards favouring environments where antibiotic resistance is key for survival. The increasing prevalence of the *A. baumannii* ST19 lineage and its antibiotic and non-antibiotic (environmental stresses and disinfectants) resistance is highly concerning and warrants greater attention in surveillance efforts.

Material and Methods

Bacterial isolates, genomes and metadata

Three isolates of *A. baumannii* ST19 were obtained from two different patients (patient 1 - wound, rectal swab; patient 2 - rectal swab). A further 156 genomes

(including 97 from Luo et al.(23)) of *A. baumannii* ST19 were retrieved from public repositories (GenBank and BIGSdb) as of March 2025.

DNA extraction and whole genome sequencing

The DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN). A rapid barcoding kit (V14) (ONT) was then used for library preparation which was then subjected to long-read whole-genome sequencing using the R10.4.1 flowcell with the PromethION (P2 Solo) platform by Nanopore.

Genome assembly and annotation

The long-read sequences were basecalled using the SUP model and adaptor and quality ($Q \leq 15$) trimmed using Porechop v0.2.2 (https://github.com/rrwick/Porechop) and NanoFilt v2.8.0 (37), respectively. The reads were then assembled using Flye2.9.3 (38).

Phylogenetic analysis

The maximum-likelihood tree was constructed by Prokka (39) generated gff files used for the core-genome alignment created by Roary (40). RAxML (41) was used for the calculation of the phylogenetic tree under GTRGAMMA model of rate heterogeneity method supported by 500 bootstraps. The tree was visualized in iTOL (42).

Identification of resistance determinants

The resistance and virulence genes were detected using ABRicate (https://github.com/tseemann/abricate) employing ResFinder (43) and VFDB 2025

(44) databases, respectively, with a 90% threshold for both gene identity and coverage. The typing of capsule-encoding loci (KL) and lipooligosaccharide outer core loci (OCL) was determined using Kaptive (45). The ABRicate (https://github.com/tseemann/abricate) was used to detect the presence of plasmid replicons using AcinetobacrPlasmidTyping database (https://github.com/MehradHamidian/AcinetobacterPlasmidTyping) (27), while the genes encoding GIs*ul2*, chlorite dismutase, formaldehyde resistance genes, *ompA* and T6SS-encoding genes were detected using a custom database.

Capsule production

The production of capsular polysaccharide was assessed as described before (6). Briefly, 1mL of overnight culture was centrifuged (2 minutes, 7000g), supernatant was removed a resuspended 1mL of PBS. Then, 875µL of the suspension was mixed with 125µL of Ludox LS colloidal silica [30% (wt/wt) suspension in H_2O] and centrifuged (30 minutes, 12000g) and the distance of the center of the band from the bottom of the microtube was measured. The measurements were taken in biological triplicate.

Biofilm quantification assay

The relative amount of biofilm was measured using a biofilm assay in a 96-well flat-bottomed polystyrene plate. The overnight cultures were adjusted to an OD_{600} of 0.05 in Luria–Bertoni medium and 200 µL of bacterial suspension were then inoculated into 12 wells per isolate and incubated statically, overnight at 37°C. The wells were then washed twice with 200 µL of PBS, air-dried, and stained with crystal violet 0.1% v/v for 15 min. Then, the crystal violet was pipetted out and wells were again washed twice with 200 µL of PBS and air-dried. The stain was then solubilised

with ethanol 95% v/v for 5 min and transferred to a new 96-well plate and measured in a plate reader (Cytation, Agilent BioTek) at OD_{570}. Each experiment was performed in biological triplicate.

Galleria mellonella in vivo **infection model of virulence**

The *G. mellonella* infections assay was performed as previously described (46). Briefly, an overnight culture was washed with physiological saline (PS) (0.9% NaCl) and diluted to known Colony Forming Unites (CFU) at OD_{600}1. Then approximately 2 × 10^7 CFU/mL of *A. baumannii* in 10 µL of PS was injected in the last left proleg using a 0.3 mL insulin syringe (BD MicroFine). Each strain was injected into 10 larvae, and 10 larvae were injected with PS as a negative control. The experiments were carried out in duplicates, and the survival of larvae was visualized in Kaplan-Meier curve in a time span of 72 hours.

Data availability

The whole genome sequencing data were deposited in GenBank under following accession numbers; strain 0788 - SAMN46706486, strain 0482 - SAMN48114201and strain 0604 - SAMN46706488.

Ethical statement

The clinical isolates sequenced in this study were obtained within retrospective study conducted under the ethical approval granted by the C. H. U. Brugmann CE 2023/51.

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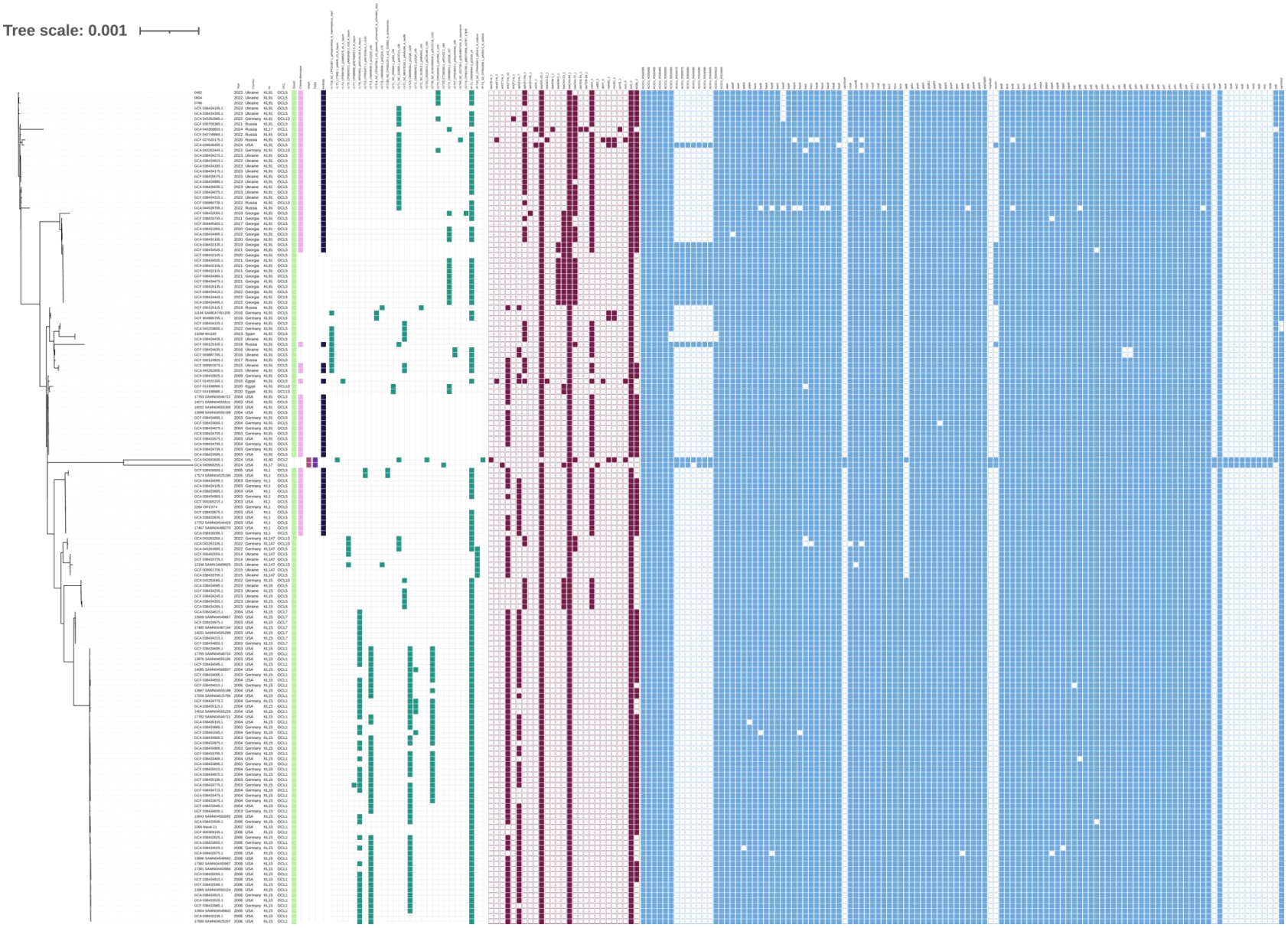
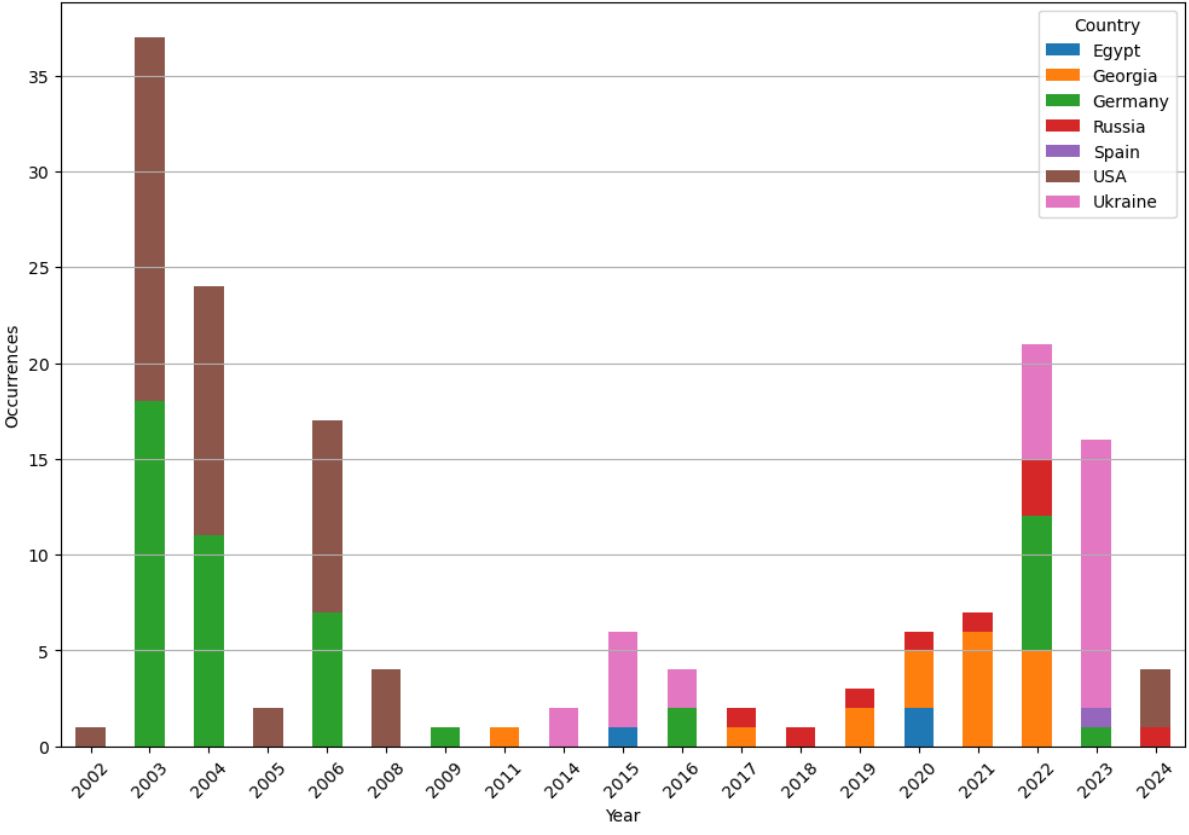
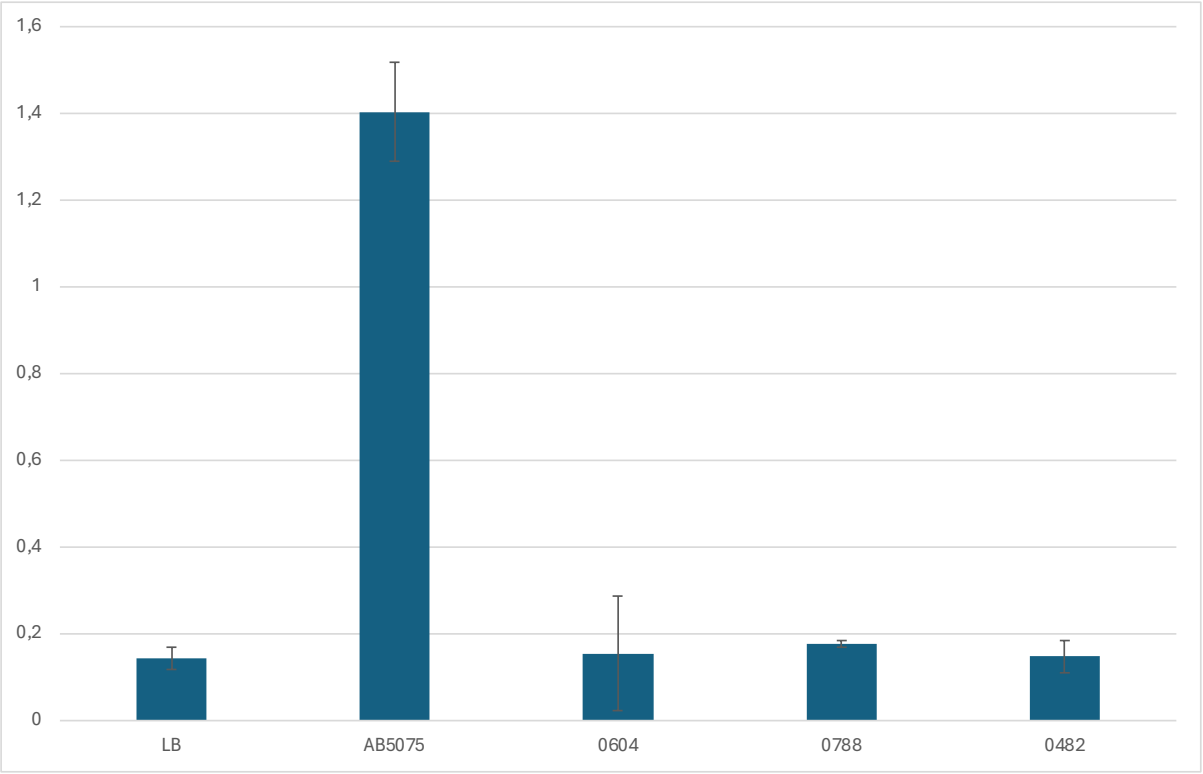
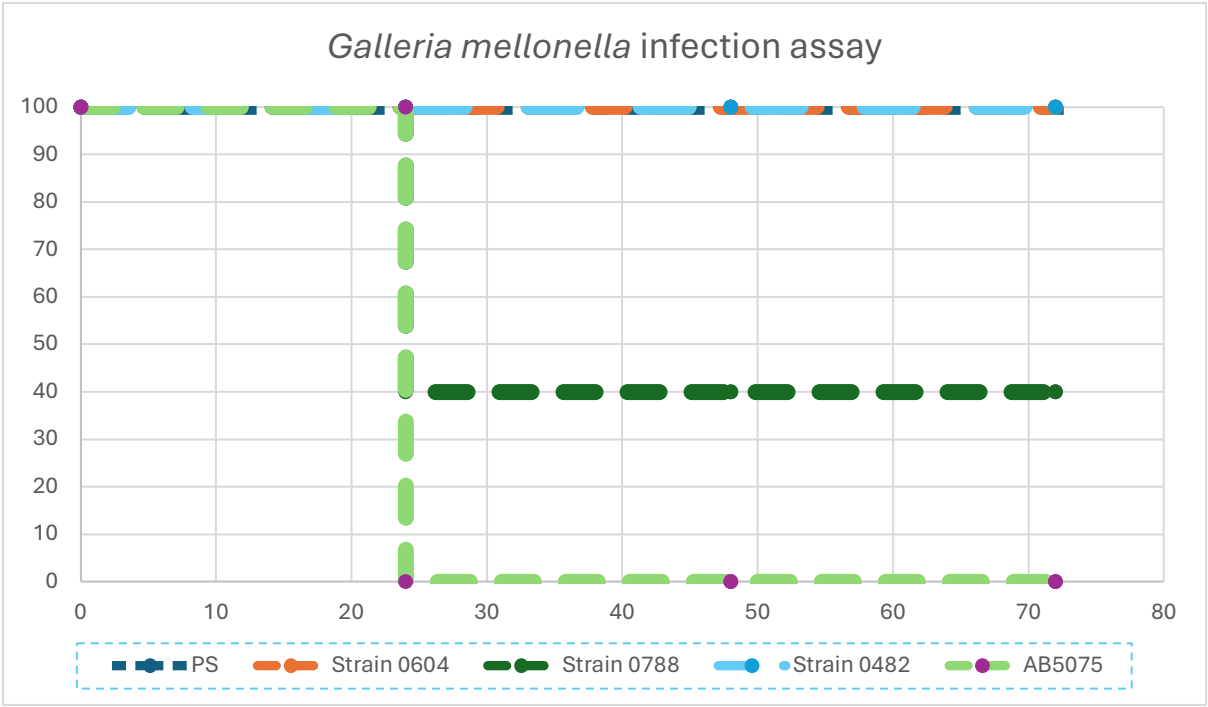

Tree scale: 0.001

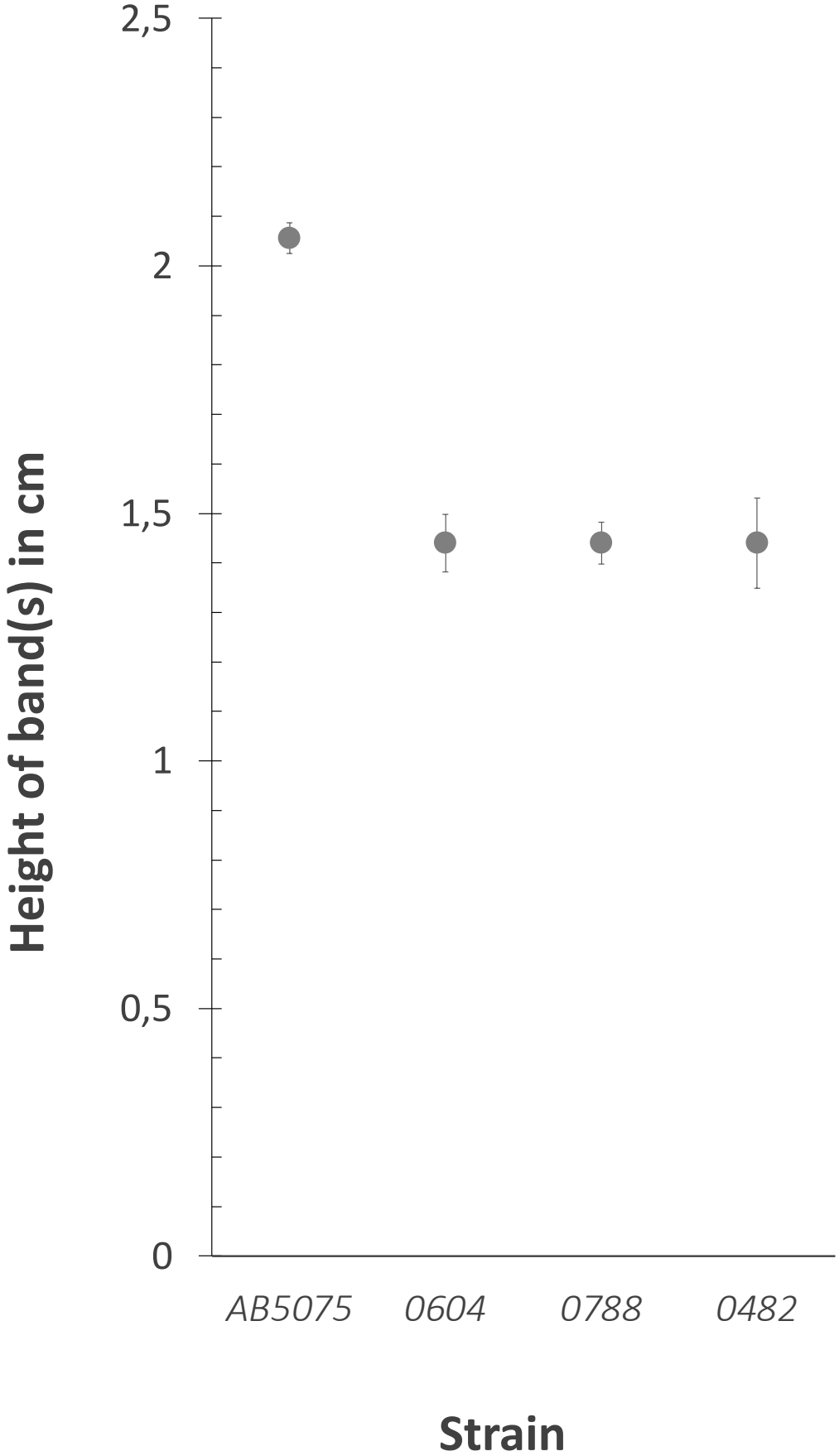
Figure 2: Phylogenetic analysis of 3 isolates and further 156 of *A. baumannii* ST19 genomes, depicting year of isolation, country, capsule locus type (KL), outer core lipooligosaccharide type (OCL), and presence of GI*sul2* genetic island, chlorite dismutase, resistance, T6SS, formaldehyde, plasmid replicons, resistance, and virulence genes, respectively.

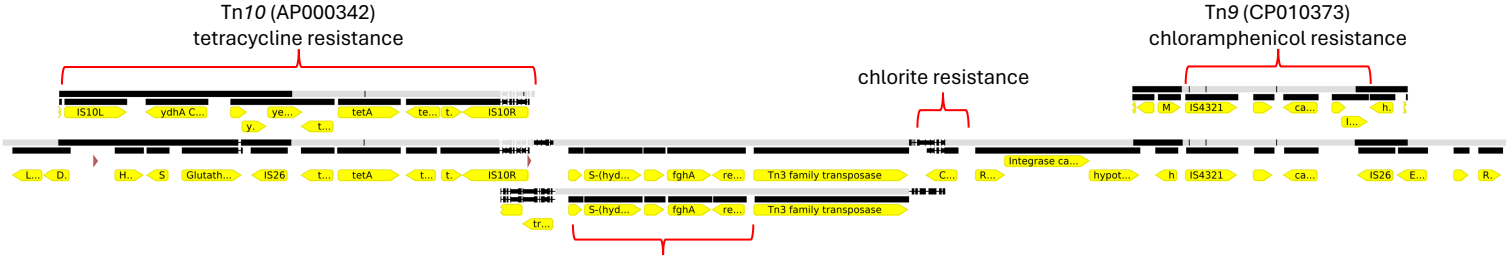

Country
Egypt
Georgia
Germany
Russia
Spain
USA
Ukraine
Occurrences
0
5
10
15
20
25
30
35
Year
2002
2003
2004
2005
2006
2008
2009
2011
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024

Tree scale: 0.001


1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
LB
AB5075
0604
0788
0482


Galleria mellonella infection assay
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
PS
Strain 0604
Strain 0788
Strain 0482
AB5075


2,5
2
1,5
1
0,5
0
Height of band(s) in cm
AB5075
0604
0788
0482
Strain


Tn*10* (AP000342)
tetracycline resistance
Tn9 (CP010373)
chloramphenicol resistance
chlorite resistance
Δ T6SS
A. baumannii
ST19
IS10L
ydhA C...
ye...
tetA
te...
t.
IS10R
y..
t...
L...
D.
H..
S
Glutath...
IS26
t...
tetA
t...
t.
IS10R
tr...
S-(hyd...
fghA
re...
Tn3 family transposase
C...
R...
Integrase ca...
hypot...
h
IS4321
ca...
IS26
E...
R.
M
IS4321
ca...
h.
I...
formaldehyde resistance
(LC589173)