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Emergence of ADC-5 Cephalosporinase in environmental *Acinetobacter baumannii* from a German tank milk with a novel Sequence Type

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Abstract

Bacteria resistant to antibiotics arguably pose the greatest threat to human health in the twenty-first century. One such bacterium that typifies antibiotic resistance is *Acinetobacter baumannii*. Frequently, hospital strains of *A. baumannii* display multidrug resistant (MDR) or extensively drug resistant (XDR) phenotypes, often requiring the use of last resort antibiotics for treatment. In addition to hospital settings, *A. baumannii* has been isolated from many highly divergent sources including wastewater treatment plant effluent, soil, and agricultural run-off with global distribution. However, such isolates remain poorly characterized. In this study, we characterized a strain of *A. baumannii,* AB341-IK15, isolated from bulk tank milk in Germany that demonstrated resistance to ceftazidime and intermediate resistance to ceftriaxone and piperacillin/tazobactam. Further genetic characterization identified an ADC-5 cephalosporinase, first incidence in an environmental isolate; and an OXA-408 oxacillinase that may contribute to this phenotype. Interestingly, AB341-IK15 is of a novel sequence type. This research underscores the importance of studying isolates of *A. baumannii* of non-clinical origin to understand the antibiotic resistance and virulence potential of environmental isolates of *A. baumannii* as well to understand the diversity of this species.

DATA SUMMARY

Data for strain AB341-IK15 has been deposited in the NCBI's Genbank repository under the biosample SAMN26898556, accession number JANBMU000000000.

INTRODUCTION

Acinetobacter baumannii is the World Health Organization's top priority organism for which new antibiotics are critically needed [1]. Understanding ways by which *A. baumannii* acquires resistance to antibiotics are critical for the design of effective therapeutic interventions. *A. baumannii* has been historically isolated from hospital settings and much work has been done in clinical strains [2–6]. Up to 70% of strains are multi-drug resistant (MDR) with many resistant to last resort antibiotics such as colistin and carbapenems [7]. Further, *A. baumannii* possesses various virulence factors such as biofilm formation, motility, secretion of proteases and iron acquisition systems that allow it to persist and thrive in a nutrient poor host environment leading to its success as a pathogen [8]. While clinical isolates of *A. baumannii* have been described extensively, there are limited studies characterizing environmental isolates of *A. baumannii*. However, recently it was suggested that this species needs to be considered a One Health problem, as several isolates from animals and plants belong to novel Sequence Types (ST) and have clinically relevant antibiotic resistance genes [9]. Thus, the study of environmental isolates of *A. baumannii* is necessary to identify the reservoirs of antibiotic resistance determinants and even novel lineages in this species. In this study, we characterize an environmental isolate of *A. baumannii*, AB341-IK15, that was isolated

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Keywords: antibiotic resistance; beta-lactamase; food source; human health ; pathogen.
Abbreviations: ANI, average nucleotide identity; ARG, antibiotic resistant gene; CLSI, clinical laboratories standards institute; ST, sequence type.
NCBI Genbank repository biosample SAMN26898556, accession number JANBMU000000000.
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Impact Statement

Acinetobacter baumannii is one of the most important nosocomial pathogens that infects individuals with a weakened immune system and is difficult to treat with current antibiotics. However, not much is known about strains prevalent in various environmental niches. In this study, we have isolated a strain from tank milk that harbours an antibiotic resistance gene that confers resistance to a particular antibiotic class; the β-lactams. This is the first time this gene has been found in an environmental isolate of *A. baumannii*. Furthermore, we show that this isolate represents a novel sequence type that has never been seen before, implying that this environmental strain is different from hospital strains. These findings show that environmental strains are significantly diverse and harbour antibiotic resistance genes which could impact treatment success of *A. baumannii* infection in the hospital.

from bulk tank milk in Alsfeld, Germany using *Acinetobacter* spp. selective CHROMagar (CHROMagar, Paris, France) at 37°C. It is not uncommon to isolate *Acinetobacter* spp. from dairy processing environments, as it is one of the top 25 most abundant and prevalent genera in pasture and feed, farm environments, teat skin, teat and bulk tank milk [10]. Accumulation of large numbers of antibiotic resistance genes (ARGs) are typical of *A. baumannii* isolates [11] and are of great concern as many are resistant to last resort antibiotics such as carbapenems and colistin [1]. Environmental isolates are also known to harbour such resistance mechanisms and may act as ARG reservoirs [12, 13]. Therefore, the purpose of this study was to characterize AB341-IK15 to identify determinants of antibiotic resistance and virulence present in this strain.

IDENTIFICATION OF *ACINETOBACTER* SPP.

Species misidentification is prominent within the *Acinetobacter* genus due to the large versatility and diversity within a single species combined with the fact that there is no simple technique for accurate identification. Phylogenetic markers and Average Nucleotide Identity (ANI) have been suggested as a more accurate way to identify *Acinetobacter* spp. [14]. Thereby, it is best to use a multi-pronged approach for identification and genomic characterisation of new *A. baumannii* isolates. For genotypic characterisation, a DNAeasy UltraClean microbial kit (Qiagen, MD, USA) was used to extract genomic DNA from a purified colony of AB341-IK15 according to the manufacturer's instructions. Sequence libraries were prepared and pooled using the DNA prep and the NextSeq 500 mid output reagent kits (Illumina, CA, USA). Illumina NextSeq 500 platform, at the AAFC-ORDC, was used for whole-genome sequencing and *de novo* assembled using SPAdes v. 3.12.0 [15]. Quality assessments were performed using QUAST v 5.0.2 [16] and CheckM v1.0.11 [17] with a 95% completeness and equal or less than 5% contamination accepted, with AB341-IK15 meeting these criteria. The sequence has been deposited to the NCBI Genbank (biosample SAMN26898556 and accession JANBMU000000000). AB341-IK15 was identified as *A. baumannii* based on the ANI. To evaluate the relatedness of AB341-IK15 to other strains of *A. baumannii,* its sequence type (ST) was determined using the MLST pipeline [18] via the Pasteur scheme [19] which made use of PubMLST [20]. AB341-IK15 was found to be of a novel ST. The relationship of AB341-IK15 to each of the international clones (ICs) was evaluated using a phylogenomic approach. The allelic profiles of each IC as well as AB341-IK15 were established using the same method as AB341-IK15. After individual genes alignments were created using Clustal Omega 1.2.2 [21], sequences were concatenated using the index function in Geneious Prime and phylogenetic tree generated using RAxML v.8.2.11 [22] with a GAMMA model of rate heterogeneity and a maximum likelihood estimate of the alpha-parameter. As shown in Fig. 1, AB341-IK15 is most closely related to a group formed by IC4, IC5, and IC6, although it is on a different branch separate from all three of these. Characteristic profiles based on capsular polysaccharide (KL) type and lipopolysaccharide outer core (OCL) type are also actively used to track specific lineages of concern. Kaptive [23], a database originally developed for capsule typing for *Klebsiella pneumoniae*, has recently been supplemented with a database specific for *A. baumannii* [24]. Typically, this database uses a minimum threshold of 'good', meaning that the locus of interest was found in a single piece or with ≥95% coverage, with ≤3 missing genes and ≤1 extra gene. These thresholds did not yield any results for AB341-IK15. However, using the loosest parameters, Kaptive determined that AB341-IK15 is within the KL95 and OCL22 lineages. Further work needs to be done with regards to the significance of these assignments as AB341-IK15 may represent novel alleles of these KL and OCL types. This exemplifies the diversity of *A. baumannii* and not only the emergence of novel lineages within the clinical setting [25] but also in the environment.

IDENTIFICATION OF ADC-5 CEPHALOSPORINASE

AB341-IK15 was tested for susceptibility to antibiotics. Using the Clinical Laboratory Standards Institute (CLSI) [26] broth microdilution method, the CANWARD panel of antibiotics were tested [27]. AB341-IK15 is susceptible to most antibiotics (Table 1). However, using CLSI breakpoints, it displays resistance to ceftazidime and intermediate resistance to ceftriaxone as well as to piperacillin/tazobactam.

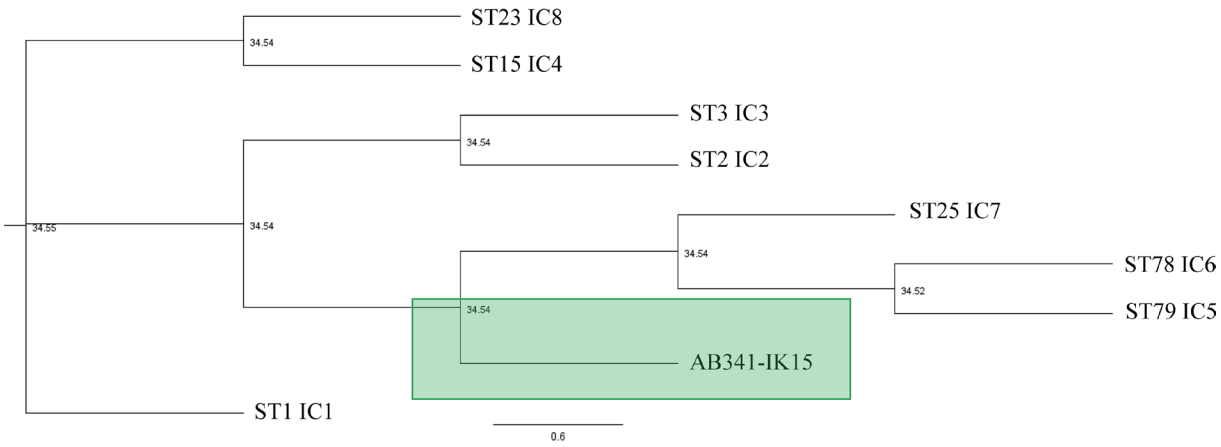

Fig. 1. Phylogenetic relationship between Sequence Types (International Clones- IC) and AB341-IK15. Representative assemblies from each IC were used for this analysis and are as follows: IC1- SAMN07257378, IC2 – SAMN09667773, IC3- SAMN01828181, IC4 – SAMN09951355, IC5 – SAMN09951336, IC6 – SAMN12509149, IC7 – SAMN09951357, IC8 – SAMN03069270. The Pasteur Multi-locus Sequence Type (MLST) profiles of each IC were obtained, as well as the profile for AB341-IK15 using the PubMLST online webtool. The sequences for these seven genes were aligned using Clustal W and then concatenated using Geneious Prime. RAxML generated the phylogenetic tree. AB341-IK15 is highlighted in the green box and is in closest relation to ICs 5, 6 and 7 although is found in a clade all of its own.

In addition to susceptibility testing, further bioinformatic investigation analysed the AB341-IK15 scaffold using the Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD) [28], via the ABRicate pipeline [29] in October 2022 considering perfect, and strict hits. The observed resistant phenotype to ceftazidime and intermediate resistance to ceftriaxone and piperacillin/tazobactam may be explained by the presence of an AmpC β-lactamase without carbapenemase activity, sharing 96% identity to bla_{ADC-5} (Genbank accession AJ575184) as well as a β-lactamase of 98.6% identity with $bla_{OXA-408}$ (Genbank accession KJ584917) (Fig. 2). ADC-5 is a chromosomally encoded cephalosporinase originally identified in a clinical strain of *A. pittii* [30] and has only been characterized in MDR clinical isolates [31]. To the best of our knowledge, this is the first report of ADC-5 in an environmental isolate of *A. baumannii*. The nucleotide sequence of AB341-IK15 ADC-5 was translated using the ExPASy translate tool [32], aligned with AJ575184 using MAFFT with the G-INSi iterative refinement method [33], and analysed using ESPript (https://espript.ibcp.fr) [34] (Fig. 3a). The mutations observed in AB341-IK15 ADC-5 namely, Q163K and T264N, have been previously characterized via expression in *E. coli* and these mutations individually, are linked with a decrease in susceptibility to ceftriaxone, among other cephalosporins [35]. These mutations may explain the intermediate resistance phenotype observed in AB341-IK15. Furthermore, G99A, K121R, V286L, G287E, and K383E appear to be novel mutations. Using Phyre2 [36], ADC-5 (Genbank accession AJ575184) was modelled and the structure processed in EZMol v2.1 [37] to highlight the mutations in ADC-5 from AB341-IK15. Based on this analysis it can be seen that G99A is located in the H2 α helix (Fig. 3b, in yellow) which is known to be critical for β-lactamase activity [38]. Whereas, K121R, shown in green, contributes to formation of the binding pocket [39]. The impact of these mutations requires further investigation.

INVESTIGATION OF OXA-408 OXACILLINASE

OXA-408 is an intrinsically encoded oxacillinase and falls into the largest family of β-lactamases, the OXA-51-like [40]. Only two members of the family have been biochemically characterized and have a role in carbapenem resistance [41]. Modelling studies predict that ceftazidime is the strongest binding substrate for OXA-51-like β-lactamases [42] suggesting that OXA-408 in AB341-IK15 likely contributes to the clinical resistance phenotype. OXA-408 was originally identified in *A. baumannii* from a dog rectum in Zgharta, Lebanon [40] and therefore it is not necessarily unique to non-clinical settings. The use of third and fourth generation cephalosporins in agricultural practice in Germany has decreased by 52.64% from 2011 to 2018 but hesitancy in compliance has made any further decrease difficult [43]. The continued use of cephalosporins in agriculture provides a likely explanation of the emergence of intrinsic resistance mechanisms such as ADC-5 and OXA-408 in *A. baumannii* isolates like AB341-IK15.

Comparing the CARD profile of AB341-IK15 with the type strain ATCC17978 (Genbank Accession NZ_CP018664) [44], as well as the hypervirulent, XDR hospital strain AB030 (Genbank Accession NZ_CP009257) [4], the differences between these strains are apparent (Fig. 2). Differences in the susceptibility profiles of ATCC17978, AB030, and AB341-IK15 are shown in Table 1. Comparable to ATCC17978, AB341-IK15, is susceptible to most antibiotics but differs by the lack of *adeAB, adeRS* and $bla_{OXA-259}$ which are present in ATCC17978 (Fig. 2). The absence of homologues in AB341-IK15 was validated using a manual BLASTN and tBLASTn search [45] using the specific gene entry in the CARD as the query. Although the BLASTN results validated the absence of these genes, upon

Table 1. Antibiotic susceptibility of AB341-IK15. Susceptibility testing results for the CANWARD panel of antibiotics according to the Clinical Laboratory Standards Institute (CLSI) broth microdilution guidelines. AB341-IK15 is resistant to CAZ as indicated in bold and has an intermediate susceptibility to CRO and TZP as indicated in the underlined text. All values that indicate resistance are highlighted in bold text and those that indicate intermediate resistance are underlined. Data is displayed based on three biological replicates

Strain	AMK	CFZ	FEP	FOX	CAZ	BPR	C/T	CRO	CIP	CLR	CLI	CST	DAP	DOX	ETP	GEN	IPM	LZD	MEM	NIT	TZP	TOB	SXT	VAN
ATCC17978	1	>128	4	>32	8	0.5	2	16	0.25	32	>8	2	>16	0.25	4	1	0.25	>16	0.5	>512	4	1	4	>32
AB030	**64**	>128	**>64**	>32	**>32**	>32	32	**>64**	**>16**	>32	>8	0.5	>16	**32**	>32	**>32**	**>32**	>16	**>32**	>512	**>512**	**64**	>8	>32
AB341-IK15	2	>128	4	>32	**32**	0.5	4	16	0.25	32	>8	1	>16	1	4	≤0.5	0.5	>16	0.5	512	32	≤0.5	≤0.12	>32

AMK, Amikacin; BPR, Ceftobiprole; CAZ, Ceftazidime; CFZ, Cefazolin; CIP, Ciprofloxacin; CLI, Clindamycin; CLR, Clarithromycin; CRO, Ceftriaxone; CST, Colistin; C/T, Ceftolazane/tazobactam; DAP, Daptomycin; DOX, Doxycycline; ETP, Ertapenem; FEP, Cefepime; FOX, Cefoxitin; GEN, Gentamicin; IPM, Imipenem; LZD, Linezolid; MEM, Meropenem; NIT, Nitrofurantoin; SXT, Trimethoprim/Sulphamethoxazole; TOB, Tobramycin; TZP, Piperacillin/Tazobactam; VAN, Vancomycin.

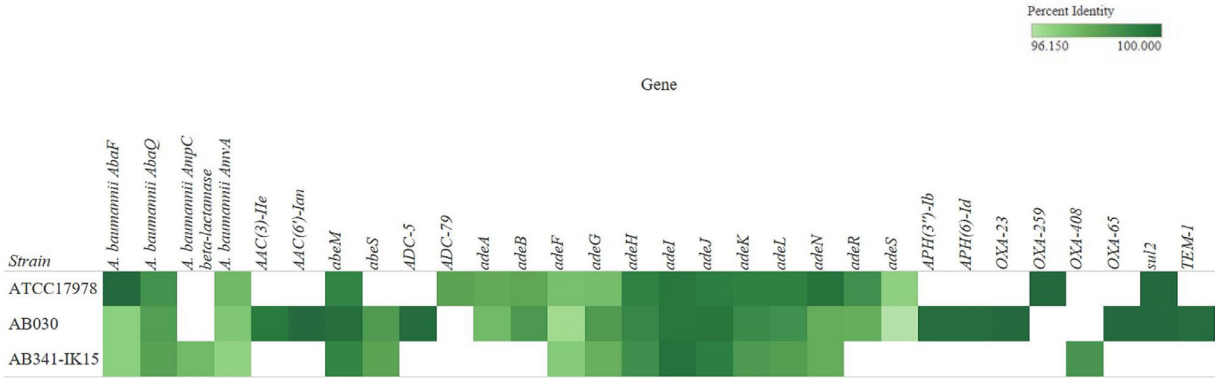

Fig. 2. Percent identity of antibiotic resistance genes in AB341-IK15 and ATCC17978. Antibiotic resistance genes (ARGs) as determined using the Resistance Gene Identifier (RGI) in the Comprehensive Antibiotic Resistance Database (CARD) from February 2022. Only those hits matching the strict (95%–99%) and perfect (>99%) criteria are shown. Note that there are two copies of OXA-23 encoded in AB030, both with 100% identity but with only one copy represented. Differences between ATCC17978 and AB341-IK15 include the presence of ADC-5 and OXA-408 in AB341-IK15 as well as a lack of *adeAB, adeRS*, OXA-259 and *sul2* compared to ATCC17978. Comparison with AB030 yields greater differences, but with the common presence of *abeS*. Upon further investigation, the *A. baumannii* AmpC beta-lactamase only observed in AB341-IK15 was determined to be an ADC-5 cephalosporinase based on sequence identity.

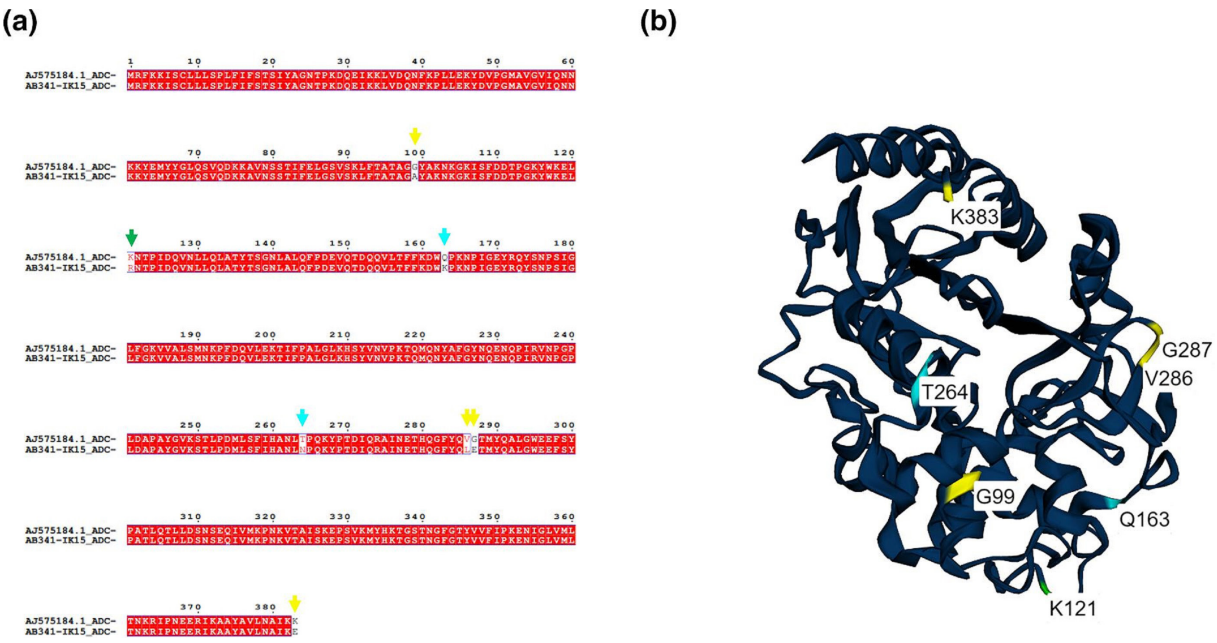

Fig. 3. Nucleotide sequence of ADC-5 and its predicted structure. Alignment of putative ADC-5 cephalosporinase from AB341-IK15 and characterized ADC-5 AJ575184 (a). Structural predictions were done after amino acid alignment in MAFFT with ESPript. Characterized mutations are denoted with cyan arrows while novel mutations are highlighted with yellow arrows and K121R is highlighted with a green arrow as it is located in the binding pocket of ADC-5. Structural prediction of putative ADC-$5_{AB341\text{-}IK15}$ using previously described ADC-$5_{AJ575184}$, mutations in ADC-$5_{AB341\text{-}IK15}$ are highlighted (b). Characterized mutations are highlighted in cyan while novel mutations are in yellow and green. G99A is located in the H2 α helices and K121R is a residue that makes up the binding pocket (highlighted in green).

further investigation using tBLASTn, homologues based on amino acid sequences were found. The tBLASTn search resulted in a hit for AdeA with a percent identity of 84.38% with 92% coverage, AdeB with 87.49% identity and 99% coverage, AdeR showed an 80.42% identity with 97% coverage, AdeS demonstrated 65.72% identity with a coverage of 97% and OXA-259 with a 97.08% identity with 100% coverage. These results suggest that putative alleles of these genes exist and require further investigation. Additional genotypic validation was performed via RT-qPCR using the Purelink RNA Extraction and DNase treatment kits as well as the VILO cDNA synthesis kit (Invitrogen, Waltham, USA) and SYBR green master mix (Applied Biosciences, Waltham, USA) using the StepOnePlus

qPCR thermal cycler (Applied Biosciences, Waltham, USA). Relative normalized expression was calculated using the Pfaffl method with 16S rRNA as the reference gene and ATCC17978 as the reference strain [46]. In AB341-IK15, there was no detectable expression of *adeB* corroborating the genetic analysis performed with the RGI in the CARD (data not shown). The overexpression of *adeABC* is associated with aminoglycoside resistance in clinical isolates [47] and our data supports the clinical relevance of AdeABC due to the lack of homologues of this efflux pump in AB341-IK15 as well as the high degree of susceptibility to aminoglycoside antibiotics (Table 1). In contrast, AB030 and AB341-IK15 both show presence of the small multidrug resistance (SMR) family efflux pump, *abeS*. This is the only characterized member of the SMR family in *A. baumannii* and has been shown to play a minor role in susceptibility to chloramphenicol, fluoroquinolones, erythromycin and novobiocin [48]. Interestingly, AbeS pump was found to be upregulated when *A. baumannii* is exposed to colistin [49]. To what degree it plays a role in AB341-IK15 susceptibility needs to be studied further.

DIVERGENT *PILA* GENE

In addition to ARGs, *A. baumannii* employs a multitude of virulence mechanisms including biofilm formation, motility, and protease secretion which provide advantages for survival in harsh conditions as well as persistence during an infection [8]. To investigate the virulence potential of AB341-IK15, again via the ABRicate pipeline accessed in May of 2022 (Seeman n.d.) the Virulence Finder Database (VFDB) [50] was used to classify such putative genes using a threshold of 80% identity and 80% coverage. These can be observed in Fig. 4. Immediately obvious is the presence of a gene homologous to *pilA* in AB341-IK15. Upon further validation via BLASTN, the AB341-IK15 putative *pilA* gene has 99.32% identity to the gene found in *A. baumannii* ACICU (ACICU_RS16915, Genbank Accession CP000863) while ATCC17978 and AB030 have only 23 and 22% coverage, respectively, compared to ACICU,

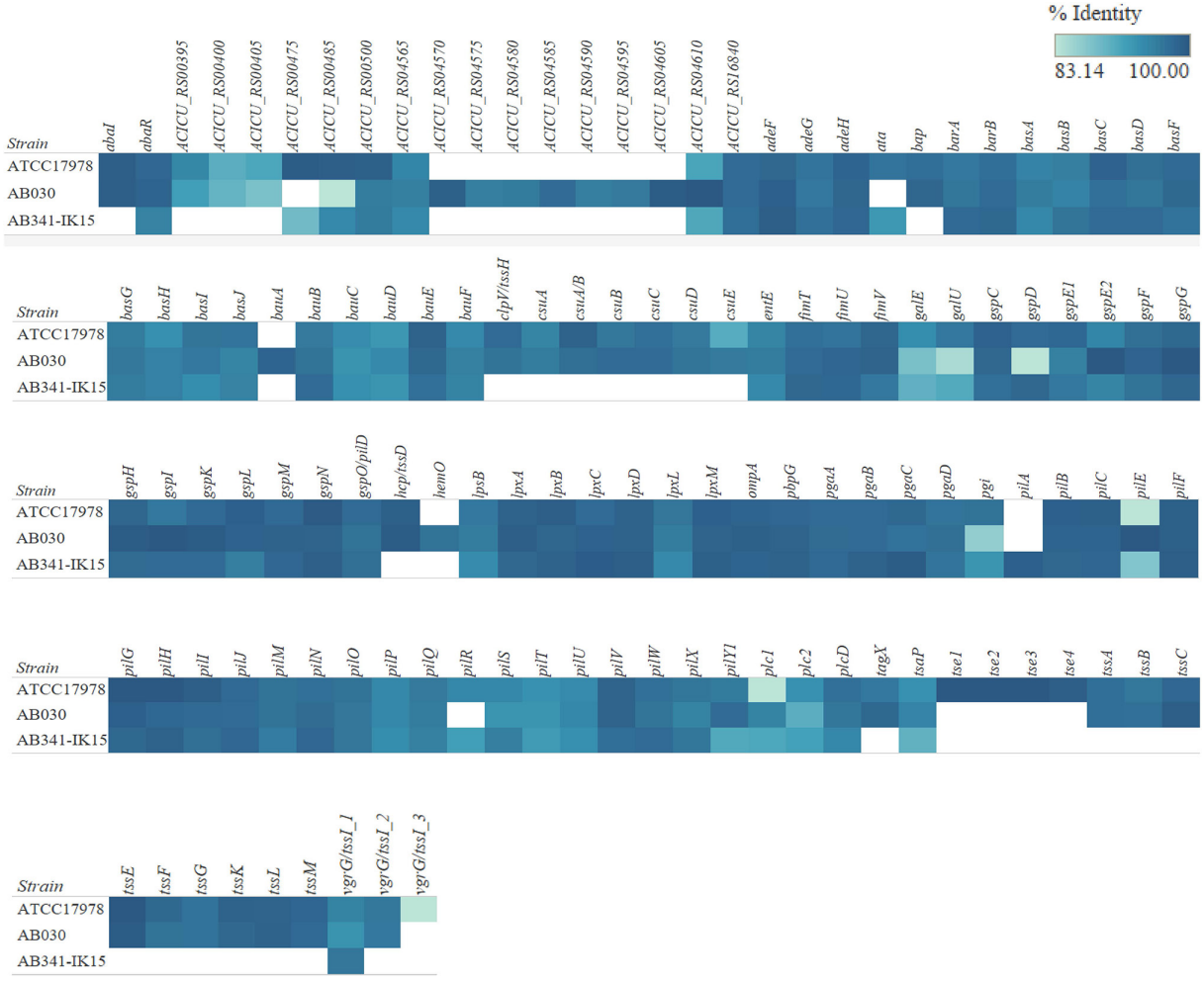

Fig. 4. Virulence analysis of ATCC17978, AB030 and AB341-IK15. Analysis was carried out using the Virulence Finder Database in May 2022. All hits from the database are shown using a cutoff of 80% identity. The percent identity match is shown in gradient shades of blue with the darker colour highlighting a closer match to the database. If there is more than one copy of each gene, these are signified by a numerical value after the gene name indicating the copy number. AB341-IK15 contains a homologue of pilA involved in motility and biofilm formation in *A. baumannii*.

supporting the fact that these *pilA* genes are highly divergent from that of ACICU. PilA is a part of the type IV pili (T4P) assembly in *A. baumannii* [8]. T4P is involved in virulence phenotypes such as motility [51], natural transformation [52] and biofilm formation [53]. In *A. baumannii*, PilC is a platform protein that interacts tightly with the extension ATPase, PilB, and the retraction ATPase, PilT. PilA is the major pilin subunit, and upon assembly with other PilA subunits, forms the functional pilus. The *pilA* gene shows high divergence within the species [8, 53]. Comparison of *pilA* from clinical isolates ACICU, AB5075 and BIDMC57 demonstrated that glycosylation and other biochemical differences resulted in an inverse relationship between biofilm formation and motility [53] as observed in *Pseudomonas aeruginosa* [54]. This led to an investigation into the motility and biofilm formation capabilities of AB341-IK15. Using minimal motility media with 0.3% agarose, overnight cultures of AB341-IK15, ATCC17978 and AB030 were normalized to an A_{600} of 1.0 and then 3 µl of the culture was stab inoculated into the centre of the plates. After incubation at 37 °C for 18 h, the diameter of the distance travelled was measured across three locations, and then averaged [55]. An ordinary one-way ANOVA test was applied for statistical analysis using GraphPad Prism v.9.3.1. Interestingly, in comparison to ATCC17978, a hyper-motile strain, AB341-IK15 appears to be non-motile under the conditions tested (Fig. 5a). Previous studies have shown the induction of motility in non-motile strains under conditions without stressor molecules such as sodium chloride [56], so it is possible that AB341-IK15 is motile under such other conditions as well but this needs to be investigated further. Biofilm formation was evaluated using a modified protocol [55], 96 well flat bottom plates were inoculated with 150 µl cultures standardized to A_{600}=0.005 and incubated at 37 °C for 48 h. After which, planktonic cells were removed via washing with mQH_2O and then the biofilm was stained with 0.1% crystal violet for 30 min. Removal of the stain, followed by dissolution with 30% acetic acid then allows for the measurement of the solubilized biofilm at A_{550}. AB341-IK15 exemplifies this inverse relationship between biofilm formation and motility, being non-motile (Fig. 5a) while forming quantitatively more biofilm than either ATCC17978 or AB030 (Fig. 5b). Considering AB341-IK15 *pilA* is highly similar to ACICU *pilA*, the virulence potential of AB341-IK15 could be considerable. ACICU is predicted to participate in trans-bundling of its pili with other cells in the vicinity and is thereby able to better form microcolonies leading to an increased ability to form biofilms [53] and this may in fact be the case with AB341-IK15. Further investigation into *pilA* in AB341-IK15 is ongoing.

SUMMARY

In summary, evaluation of the ARG and susceptibility profiles of environmental isolates is vital to better understand the resistance potential of *A. baumannii*. AB341-IK15 represents an isolate of novel ST supporting the fact that the diversity of the species continues to expand. This isolate serves as an example where non-clinical isolates of *A. baumannii* not only harbour antibiotic resistance gene(s) but also display resistance to antibiotics. Thus, our work contributes towards the knowledge base to fully understand the diversity of *A. baumannii*. Notably, this is the first time the cephalosporinase ADC-5 has been identified in an environmental *A. baumannii* isolate.

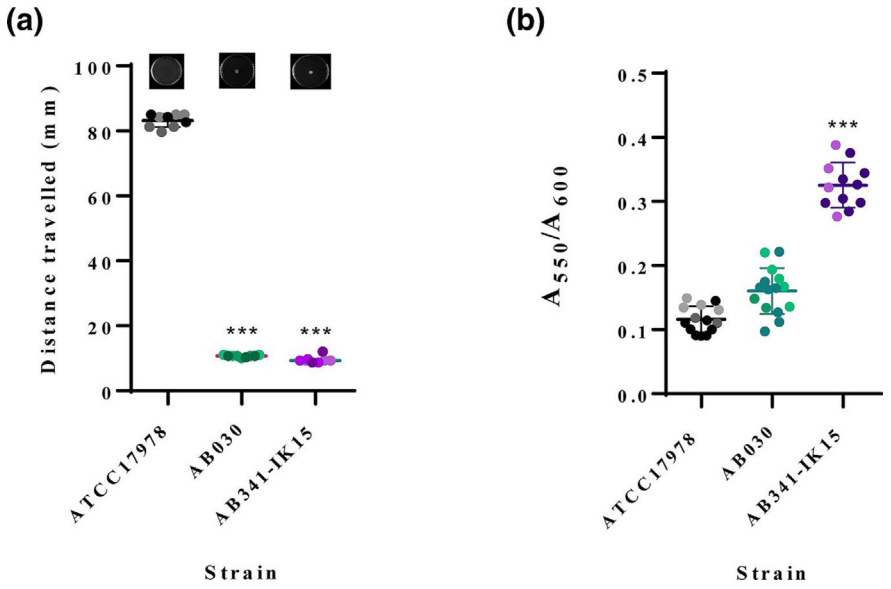

Fig. 5. Motility and biofilm formation by ATCC17978, AB341-IK15 and AB030. ATCC17978 is a hyper-motile strain while AB341-IK15 and AB030 are non-motile (a). Motility experiments were performed with three technical replicates for each of three biological replicates. Biofilm formation quantification of ATCC17978, AB030 and AB341-IK15 (b). AB341-IK15 produces more biofilm than the type-strain ATCC17978 and the MDR clinical isolate AB030 as determined by the crystal violet staining assay. The data has been normalized to the OD_{600} of each strain to account for differences in cell density within the biofilm. Data was generated with five technical replicates for each of three biological replicates. Statistical analysis was performed in GraphPad Prism 9.3.1 using a one-way ANOVA.

The mutations in AB341-IK15 ADC-5 are consistent with those in the literature suggesting a decrease in susceptibility to ceftriaxone, which corresponds to what is observed phenotypically. Two additional mutations in the AB341-IK15 ADC-5 are novel and their contribution to susceptibility will be investigated in future studies. In conclusion, our study underscores the importance of studying non-clinical *A. baumannii* isolates for a better understanding of the reservoirs of resistance and virulence determinants in *A. baumannii*.

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Author contributions
Each author contributed with the following roles according to https://casrai.org/credit/guidelines: Sykes EME - Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft. M.-E.V. – Investigation, Methodology, Software, Writing – review & editing. Z.G. - Conceptualization, Resources, Writing – review & editing. D.J. – Funding Acquisition, Resources, Methodology, Writing – review & editing. C.J. – Methodology, Writing – review & editing. G.S. – Methodology, Writing – review & editing. A.Ö. –Resources 273 Khan IUH - Funding acquisition, Conceptualization, Resources, Writing – review & editing. C.-R.S. - Funding acquisition, Conceptualization, Resources, Writing – review & editing. K.A. - Funding acquisition, Conceptualization, Resources, Writing – review & editing.

Conflicts of interest
The author(s) declare that there are no conflicts of interest.

Ethical statement
This is not applicable for this study.

Consent to publish
This is not applicable for this study.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000485.v2.3
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Helina Marshall; Queen's University Belfast, UNITED KINGDOM

Date report received: 15 May 2023
Recommendation: Accept

Comments: Thank you for addressing the reviewers comments. This paper has now been accepted for publication in Access Microbiology.

SciScore report

https://doi.org/10.1099/acmi.0.000485.v2.1
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iThenticate report

https://doi.org/10.1099/acmi.0.000485.v2.2
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Author response to reviewers to Version 1

We thank both reviewers for their insightful comments. We have modified our manuscript to incorporate the comments/suggestions provided. Our point-by-point response the comments is listed below. Please find below our responses to the suggestions/comments.

Reviewer 1

Can the authors provide the KL and OCL type of the isolate, which could be beneficial and interesting information to provide.

This is a good addition to the manuscript. Thank you for your suggestion. This has been added on lines 125-134.

L30-2: Frequently, hospital strains of A. baumannii display multidrug resistant (MDR) or extreme drug resistant (XDR) phenotypes, often requiring the use of last resort antibiotics for treatment.

- XDR usually refers to extensively drug-resistant. If not the case, what is the definition of an "extreme drug resistant" phenotype?

Thank you for catching this mistake. It has been corrected in all iterations of XDR, specifically on line 33.

Unusual paragraph sectioning as the "5. Introduction" contains the entire text of the manuscript.

Our apologies for this error. We have divided the manuscript into further sections such as lines 97, 138, 172. 220 and 268.

L104-6: To evaluate the relatedness of AB341-IK15 to other strains of A. baumannii, ABRicate pipeline (Seeman 2022) and ST classification via the Oxford scheme (Bartual et al. 2005) were used.

- More suitable citation of ABRicate is "Seemann T, Abricate, Github https://github.com/tseemann/abricate" as its Github page recommends. The year "2022" is therefore irrelevant since the software is not from 2022. However, the year should be stated when referring to a particular database used via ABRicate, or the version of the database. Which database was employed in order to "evaluate the relatedness of AB341-IK15 to other strains of A. baumannii"?

Thank you for this comment. The database used was CARD within the ABRicate platform but in fact this is in the incorrect section as the ST was determined using PubMLST. This had been modified on lines 114-115, and 139 and in regard to the VFDB on line 225.

- Employing the Pasteur scheme for MLST would be beneficial as well, since the Oxford scheme detects also targets which are more prone to recombination.

Thank you for pointing this out. We realize that an error was made when writing the manuscript because all analysis was in fact performed with the Pasteur scheme. In fact the reference in the original draft of the manuscript was for Diancourt *et al*2010 which refers to the Pasteur scheme. This has been corrected on lines 115.

How were the representatives of the particular International Clones selected? Have you considered performing a phylogenetic analysis of the available isolates of the same ST (either Oxford or Pasteur if assessed) to see if you isolate clusters with other environmental or clinical isolates?

The representative of the ICs was selected based on a couple of criteria. Completeness of genome sequencing was considered along with completed metadata. As this isolate is of a novel ST, analysis to determine other isolates of a similar ST would have to be undertaken. This is a great suggestion and is being considered for our next publication.

L162-4: Missing reference.

Thank you for catching this. This has been remedied on line 183.

L164-5: Considering the degeneration of the genetic code, perhaps BLASTP should be applied on top of the BLASTN.

Thank you for your suggestion. This analysis was performed using tBLASTn (which perhaps is a better option than BLASTP) and yielded the same results as the BLASTN. The addition of this analysis can be found on lines 192-201.

L235: "FAutor"

This typo has been corrected on line 283.

Reviewer 2

Comments to Author: In this study, the authors characterized a strain of A. baumannii, AB341-IK15, isolated from bulk tank milk in Germany that demonstrated resistance to ceftazidime and intermediate resistance to ceftriaxone and piperacillin/tazobactam. Further genetic characterization identified an ADC-5 cephalosporinase, first incidence in an environmental isolate; and an OXA-408 oxacillinase that may contribute to this phenotype. Interestingly, AB341-IK15 is of a novel sequence type. This research underscores the importance of studying isolates of A. baumannii of non-clinical origin to understand the antibiotic resistance and virulence potential of environmental isolates of A. baumannii as well to understand the diversity of this species.

Problems and suggestions:

1. In line 163 and 164 "the lack of adeAB, adeRS and blaOXA-259 which are present in ATCC17978 (Error! Reference source not found.)", verify and modify that please.

Sorry about this error. This has been removed and can be found on line 192.

2. In line 235 "6. FAuthor statements" should be "6. Author statements".

This has been corrected on line 283.

3. In the author's articles, "extreme drug resident" and "Extremely Drug-Resistant" appear. It is recommended to use "Extensively Drug Resident" instead of it.

Thank you for catching this. It has been modified to extensively drug resistant on line 33.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000485.v1.7
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Helina Marshall; Queen's University Belfast, UNITED KINGDOM

Date report received: 28 February 2023
Recommendation: Minor Amendment

Comments: The work presented is clear and the arguments well formed. This is a study that would be of interest to the field and community. The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments. Please have a look at the structure, currently it only consists of Abstract, Impact Statement, Data Summary & Introduction. Please introduce Methods, Results and Discussion headers as well to improve readability. The font size in the figures is relatively small, could you increase these and also make the lines wider in the phylogenetic tree to improve readibility?

Reviewer 4 recommendation and comments

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Guerrino Macori; University College Dublin, Centre for Food Safety, Belfield, Dublin, IRELAND
https://orcid.org/0000-0001-5835-8409

Date report received: 27 December 2022
Recommendation: Minor Amendment

Comments: The manuscript presents the report of the analysis of Acinetobacter baumannii strain isolated from bulk tank milk in Germany. The isolate demonstrated resistance to ceftazidime and was genomically analysed. The study presents with methodological rigour the protocols used for the sample preparation, characterisation and the tools used for genomic analysis. The manuscript needs more structure, the title of the section introduction (line 63) is not clear, and other sections are expected also for a short report. This organization would better present the key findings. A few suggestions and points that need clarification are presented below: Line 104 - include how the species identification was predicted using ANI. Line 108 - explain the protocol (tool/software or pipeline) for the "phylogenomic approach". Line 123 - was just one scaffold used for the analysis? The results are well presented, however, some of the details on the characterisation (both phenotypical and genotypical) can be presented in a table. A clarification on the importance of the. nucleotide sequence of ADC-5 and its predicted structure is expected. Is this the key finding of the study? Why is important the alignment of putative ADC-5 cephalosporinase from AB341-IK15 and characterized ADC-5 AJ575184? How are the predicted structures based on the mutations statistically supported? The literature analysis is comprehensive. Please comment on lines 163-164 (verify the note "Error! Reference source not found").

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 3 recommendation and comments

https://doi.org/10.1099/acmi.0.000485.v1.5
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Viviane Santos de Sousa; Universidade Federal do Rio de Janeiro, BRAZIL

https://orcid.org/0000-0002-8232-9955

Date report received: 16 December 2022
Recommendation: Accept

Comments: Line 163 - there is an error with the reference. It is like: ... which are present in ATCC17978 (Error! Reference source not found.).

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 2 recommendation and comments

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Yanjiong Chen; Xi'an Jiaotong University, CHINA

Date report received: 22 October 2022
Recommendation: Accept

Comments: 1. Methodological rigour, reproducibility and availability of underlying data 2. Presentation of results 3. How the style and organization of the paper communicates and represents key findings 4. Literature analysis or discussion 5. Any other relevant comments

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

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Charles Van der Henst; Vrije Universiteit Brussel (VUB) / Flanders Institute for Biotechnology (VIB), VIB-VUB Center for Structural Biology, BELGIUM
https://orcid.org/0000-0002-3451-9439

Date report received: 03 October 2022
Recommendation: Minor Amendment

Comments: Dear, I have now reviewed the article entitled: "Emergence of ADC-5 Cephalosporinase in environmental Acinetobacter baumannii from a German tank milk with a novel Sequence Type " for publication in "Access Microbiology". This is a nicely written manuscript describing an environmental isolate of A. baumannii. The authors carried out multiple experiments and analyses to support their claims, in sound methodology. Here are my comments: Can the authors provide the KL and OCL type of the isolate, which could be beneficial and interesting information to provide. L30-2: Frequently, hospital strains of A. baumannii display multidrug resistant (MDR) or extreme drug resistant (XDR) phenotypes, often requiring the use of last resort antibiotics for treatment. - XDR usually refers to extensively drug-resistant. If not the case, what is the definition of an "extreme drug resistant" phenotype? Unusual paragraph sectioning as the "5. Introduction" contains the entire text of the manuscript. L104-6: To evaluate the relatedness of AB341-IK15 to other strains of A. baumannii, ABRicate pipeline (Seeman 2022) and ST classification via the Oxford scheme (Bartual et al. 2005) were used. - More suitable citation of ABRicate is "Seemann T, Abricate, Github https://github.com/tseemann/abricate" as its Github page recommends. The year "2022" is therefore irrelevant since the software is not from 2022. However, the year should be stated when referring to a particular database used via ABRicate, or the version of the database. Which database was employed in order to "evaluate the relatedness of AB341-IK15 to other strains of A. baumannii"? - Employing the Pasteur scheme for MLST would be beneficial as well, since the Oxford scheme detects also targets which are more prone to recombination. How were the representatives of the particular International Clones selected? Have you considered performing a phylogenetic analysis of the available isolates of the same ST (either Oxford or Pasteur if assessed) to see if you isolate clusters with other environmental or clinical isolates? L162-4: Missing reference. L164-5: Considering the degeneration of the genetic code, perhaps BLASTP should be applied on top of the BLASTN. L235: "FAutor"

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

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iThenticate report

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