Differential development of antibiotic resistance and virulence between Acinetobacter species

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2　species
3
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20

Abstract:

The two species that account for most cases of *Acinetobacter*-associated bacteraemia in the UK are *Acinetobacter lwoffii*, often a commensal but also an emerging pathogen, and *A. baumannii*, a well-known antibiotic-resistant species. While these species both cause similar types of human infection and occupy the same niche, *A. lwoffii* (unlike *A. baumannii*) has thus far remained susceptible to antibiotics. Comparatively little is known about the biology of *A. lwoffii* and this is the largest study on it conducted to date, providing valuable insights into its behaviour and potential threat to human health.

This study aimed to explain the antibiotic susceptibility, virulence, and fundamental biological differences between these two species. The relative susceptibility of *A. lwoffii*, was explained as it encoded fewer antibiotic resistance and efflux pump genes than *A. baumannii* (9 and 30 respectively). While both species had markers of horizontal gene transfer, *A. lwoffii* encoded more DNA defence systems and harboured a far more restricted range of plasmids. Furthermore, *A. lwoffii* displayed a reduced ability to select for antibiotic resistance mutations, form biofilm and infect both *in vivo* and *in vitro* models of infection.

This study suggests that the emerging pathogen *A. lwoffii* has remained susceptible to antibiotics because mechanisms exist to make it highly selective about the DNA it acquires, and we hypothesise that the fact that it only harbours a single RND system restricts the ability to select for resistance mutations. This provides valuable insights into how development of resistance can be constrained in Gram negative bacteria.

Importance:

Acinetobacter lwoffii is often a harmless commensal but is also an emerging pathogen and is the most common cause of *Acinetobacter*-derived blood stream infections in England and Wales. In contrast to the well-studied, and often highly drug resistant *A. baumannii*, *A. lwoffii* has remained susceptible to antibiotics. This study explains why this organism has not evolved resistance to antibiotics. These new insights are important to understand why and how some species develop antibiotic resistance, while others do not and could inform future novel treatment strategies.

Introduction:

Acinetobacter are Gram-negative, soil-dwelling, Gammaproteobacteria. Despite being typically found in the environment, some species within the genus also cause life-threatening human infections (1), the most clinically significant of these is *A. baumannii* which is often highly multidrug resistant (2, 3).

According to United Kingdom Health Security Agency (UKHSA), in England the most common cause of *Acinetobacter*-derived bacteraemia is *Acinetobacter lwoffii* followed by *A. baumannii* (30% and 21%, respectively) (4). *A. lwoffii* is found both in soil environments and as a common commensal of human skin (5). As well as causing bacteraemia in adults, *A. lwoffii* can cause a variety of infections, often in immunocompromised hosts and is a common cause of serious neonatal infections, which can lead to sepsis (6–9).

Both *A. lwoffii* and *A. baumannii* are found in hospitals and are resistant to desiccation, irradiation, and biocides (10, 11). However, *A. lwoffii* is generally antibiotic susceptible, in contrast to the multi-drug resistance displayed by *A. baumannii* (4). There are few studies aimed at understanding *A. lwoffii* and the reasons for its comparative sensitivity are not known.

We recently showed that the number of resistance nodulation division (RND) pumps present across the *Acinetobacter* genus varies and that *A. lwoffii* encodes fewer efflux pumps from the RND family than *A. baumannii* (1). These efflux pumps are important mediators of antibiotic resistance suggesting that their absence may contribute to the difference in susceptibility to antibiotics (12). RND pumps have also been implicated in virulence and biofilm formation (13, 14).

In this study we investigated the genomic and phenotypic differences between a range of *A. baumannii* and *A. lwoffii* strains (including clinical and type strains) to understand why two closely related species have such different responses to antibiotics. This study provides insight into the development of antibiotic resistance and differences in biology and virulence in two clinically important pathogens.

Methods:

Strains used in this study

Reference strains of *A. baumannii* AYE and *A. lwoffii* NCTC 5867 were used. In addition, representative clinical and non-clinical strains were used in this study, listed in supplementary S1. All strains were cultured in lysogeny broth (LB) (Sigma) unless stated otherwise at 37°C.

Measurement of the susceptibility of to antimicrobials

The minimum inhibitory concentration (MIC) of various antimicrobials to *A. baumannii* and *A. lwoffii* was determined using the agar dilution method (15) according to EUCAST (16). Antimicrobials tested included ampicillin (Sigma #A9393), cefotaxime (Fisher #10084487), chloramphenicol (Fisher #10368030), ciprofloxacin (Fisher #13531640), clindamycin (Generon #A10227), erythromycin (Fisher #10338080), fusidic acid (Sigma #F0881), gentamicin (Fisher #10224873), meropenem (TCI Chemicals #M2279), novobiocin (Fisher #15403619), rifampicin (Fisher #10533325) and tetracycline (Fisher #10460264).

100   Biofilm formation and susceptibility
101   The ability of *A. baumannii* and *A. lwoffii* to establish monospecies biofilms and the
102   susceptibility of these biofilms to different compounds was tested. The full methods can be
103   found in supplementary S2.
104
105   Whole genome sequence analysis
106   All available *A. lwoffii* and *A. baumannii* whole genome sequences were downloaded from
107   NCBI (41 and 6,127 respectively) on 20/03/2022. In addition, laboratory strains of both *A.*
108   *baumannii* (10) and *A. lwoffii* (8) were whole genome sequenced and assembled
109   (MicrobesNG, UK). A list of strains sequenced in this study and their assembly accession
110   numbers can be found in supplementary S3.
111
112   Quast (v.5.0.2) was used to quality check (QC) sequences and those with N50 values of
113   <30,000 and >165 Ns per Kbp were removed (17). fastANI (v.1.31) was used to determine
114   average nucleotide identity of *A. baumannii* sequences to *A. baumannii* AYE (CU459141.1)
115   and *A. lwoffii* sequences to *A. lwoffii* 5867 (GCA_900444925.1) and only sequences >95.5%
116   were kept (18). MASH (v.2.2.2) (19) was also performed to identify any duplicate assemblies
117   which were then removed using a custom R script (https://github.com/C-
118   [Connor/MashDistDeReplication/blob/master/MashDistDeReplication.R](https://github.com/C-Connor/MashDistDeReplication/blob/master/MashDistDeReplication.R)). The final quality
119   step was CheckM (v.1.1.3) (20), where sequences with >5% contamination and/or <95%
120   completeness were removed. The final number of *A. baumannii* and *A. lwoffii* sequences was
121   4,809 and 38 respectively.
122
123   Assemblies were searched for antibiotic resistance genes (ARGs) (Comprehensive Antibiotic
124   Resistance Database (21)), type IV pilus genes ('twitching' database using Ref. (22)), plasmid
125   *rep* genes (database from Ref. (23)) and virulence and biofilm genes ('vandb' database using
126   Ref. (24)) using ABRicate (v.0.8.13). The 'twitching' and 'vandb' databases can be found at:
127   https://github.com/emd803/Gene-Databases/tree/main. Prophages were identified in a
128   random 10 isolates of *A. baumannii* and *A. lwoffii* using PHASTER and DNA defence systems
129   were searched for in all the genomes using DefenseFinder (v.1.0.9) (25, 26).
130
131   Selection for resistance to meropenem, ciprofloxacin and gentamicin
132   To determine if *A. baumannii* (AB18) and *A. lwoffii* (AL28) could evolve resistance to three
133   clinically relevant drugs, a selection experiment was set up, using strains clinically susceptible
134   to all three selection antibiotics. Briefly, a single colony was inoculated into 5 mL of nutrient
135   broth (Sigma) and a 1% transfer was passaged every 24 hours in increasing concentrations of
136   each drug or without drug as a control. Populations from the terminal passage were spread
137   onto LB agar and individual colonies were tested for their susceptibility to antibiotics listed
138   above, as well as moxifloxacin (Sigma #PHR1542) and ethidium bromide (Fisher #10042120).
139   Following selection, five colonies from parental strains AL28 and AB18 were subject to whole
140   genome sequencing (MicrobesNG, UK) along with two colonies that had been passaged in
141   nutrient broth only. Resulting sequences were compared to the appropriate parental strain.
142
143   Each whole genome sequence was confirmed to be from the species expected using ANI as
144   above (>95%) and sequences were compared to both the ancestral strain and the cells
145   passaged in nutrient broth only, using Snippy (v.4.6.0) to find sequence variants (27).
146

147 Measurement of twitching motility and growth
148 A previously described crystal violet assay was used to measure twitching motility in *A.*
149 *baumannii* and *A. lwoffii* (28). Additionally, growth in LB and human serum (Merck #H4522)
150 was measured. Full methods in supplementary text S4.
151
152 Scanning electron microscopy
153 Strains were grown overnight in LB, then diluted 1:50 for *A. baumannii* and 1:10 for *A. lwoffii*
154 in LB because *A. lwoffii* grows to a lower final cell density than *A. baumannii*. Strains were
155 grown to mid-log, washed with phosphate buffered saline (PBS) (Merck #D8537), and then
156 resuspended in 2.5% glutaraldehyde (Sigma #354400) to fix. Cells were imaged on an Apreo
157 2 Scanning Electron Microscope (Thermo Fisher) at 5,000x, 10,000x and 25,000x
158 magnification. Cell length analysis was performed in ImageJ (29) where the lengths of 100
159 randomly selected cells from each strain were measured.
160
161 Virulence in the *Galleria mellonella* model
162 *Galleria mellonella* larvae were injected with 10^6 bacterial cells as previously described (30)
163 and the number of live/dead larvae were quantified across 7 days.
164
165 Comparing the virulence in a macrophage cell line *in vitro*
166 Human monocyte THP-1 cell line (ATCC TIB-202) was cultured in Roswell Park Memorial
167 Institute (RPMI) Medium with GlutaMAX (Thermo Fisher #61870-010) supplemented with
168 10% heat-inactivated fetal bovine serum (Life Technologies, #A5256701) at 37°C and 5% CO_2.
169 THP-1 monocytes were differentiated to macrophages with medium containing 50 ng/mL
170 phorbol 12-myristate 13-acetate (PMA) (Sigma #P1585) for 3 days. Cells were then left to rest
171 for 2 days by replacing the differentiation medium with complete medium without PMA.
172 Macrophages were infected as previously described (31), with a multiplicity of infection (MOI)
173 of 100. Extracellular bacteria were killed after 2 hours using gentamicin at either 100 μg/mL
174 or at 1 mg/mL for AB05. Association, invasion, and proliferation (after 6 hours) were
175 quantified. Association was determined by subtracting the number of intracellular bacteria
176 (invasion) from the total number of bacteria associated with macrophages (and within
177 macrophages).
178
179 **Results**
180 ***A. lwoffii* is more susceptible to a broad range of antibiotics than *A. baumannii***
181 Data from the UKHSA shows that *A. lwoffii* isolated from patients in England were more
182 susceptible than *A. baumannii* to gentamicin, ciprofloxacin, meropenem and colistin (4).
183 Therefore, we sought to determine if the same was true in our diverse strain collection of
184 strains for a range of antibiotics from different drug classes (Table 1). MICs were higher for *A.*
185 *baumannii* than for *A. lwoffii* for all compounds tested. EUCAST resistance breakpoints were
186 only available for ciprofloxacin (>1 μg/mL), meropenem (>2 μg/mL) and gentamicin (>4
187 μg/mL) (16). *A. lwoffii* was clinically susceptible in all instances, whereas for *A. baumannii*, all
188 but one isolate was resistant to ciprofloxacin, three of six strains were resistant to
189 meropenem, and all were resistant to gentamicin.
190
191 **Table 1** MIC values for *A. baumannii* and *A. lwoffii* (μg/mL)

Strain	AMP	CEF	CHL	CIP	CLI	ERY	FUS	GEN	MER	NOV	RIF	TET
AB05	512	>32	256	>32	128	32	128	1024	0.5	16	16	128

AB18	64	16	128	1	64	16	128	4	0.25	8	4	1
AB19	8	32	128	>32	64	32	128	16	0.25	8	4	8
AB20	32	16	128	2	64	64	64	4	>16	16	4	>128
AB25	1024	>32	256	>32	128	64	128	128	8	32	4	>128
AB27	1024	16	128	>32	32	8	64	1024	16	8	4	>128
AL04	<1	2	2	0.06	2	<0.5	16	<2	0.03	8	0.5	0.5
AL28	<1	1	1	0.06	1	0.5	4	<2	0.12	4	0.5	0.25
AL29	<1	2	1	0.06	2	0.5	8	<2	0.03	8	0.5	0.25
AL32	<1	1	1	0.03	4	0.5	8	<2	0.12	8	0.5	0.25
AL33	<1	1	1	0.06	4	0.5	8	<2	0.12	8	0.5	0.25

192 AMP - ampicillin, CEF - cefotaxime, CHL - chloramphenicol, CIP - ciprofloxacin, CLI - clindamycin, ERY -
193 erythromycin, FUS - fusidic acid, GEN - gentamicin, MER - meropenem, NOV - novobiocin, RIF - rifampicin,
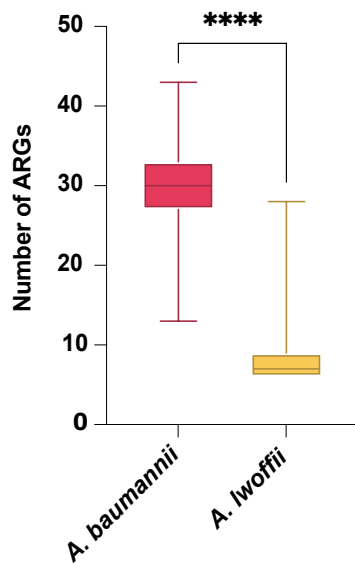194 TET - tetracycline

195

196 **A. lwoffii carries fewer antibiotic resistance genes (ARGs) than A. baumannii**
197 To explain the differences in antibiotic sensitivity between *A. lwoffii* and *A. baumannii,* whole
198 genome sequences were searched for the presence of ARGs using the CARD database.
199 Following QC there were 4,809 *A. baumannii* and 38 *A. lwoffii* genome sequences. Across the
200 *A. lwoffii* genomes 40 different ARGs were found whilst 333 different ARGs were detected
201 across *A. baumannii.* Due to the lack of available sequences for *A. lwoffii,* to quantitatively
202 compare the presence of ARGs between the two species, a random permutation was
203 conducted, which subsampled 38 sequences (the same number as the population of *A. lwoffii*
204 sequences) from the *A. baumannii* population 100 times to create an average. *A. baumannii*
205 encodes significantly more ARGs than *A. lwoffii (*p <0.0001); the mean number of ARGs in *A.*
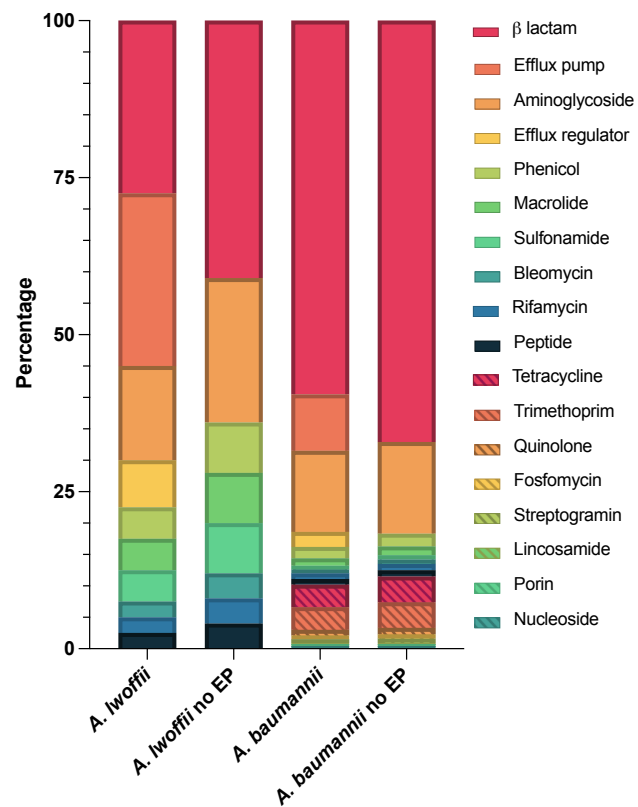206 *lwoffii* was 9 but was 30 for *A. baumannii,* Fig. 1a.

207

208 Although there was a difference in total gene presence, the classes of antibiotics that the
209 ARGs were active against was similar across the two species, Fig. 1b. The majority of ARGs
210 (>50%) found in *A. lwoffii* and *A. baumannii* reduce the host's susceptibility to beta lactams
211 and aminoglycosides.

a

b

Legend (right of b):
- β lactam
- Efflux pump
- Aminoglycoside
- Efflux regulator
- Phenicol
- Macrolide
- Sulfonamide
- Bleomycin
- Rifamycin
- Peptide
- Tetracycline
- Trimethoprim
- Quinolone
- Fosfomycin
- Streptogramin
- Lincosamide
- Porin
- Nucleoside

212

213 **Fig. 1** *A. baumannii* encodes more ARGs than *A. lwoffii*. a - Number of ARGs found per whole genome
214 sequence from either species. *A. baumannii* in pink n= 4,809, *A. lwoffii* in yellow n=38. A random
215 permutation and Welch's T test was performed to compare the average number of genes when the
216 sample sizes were the same - ****, p<0.0001. b – stacked bar chart showing drug classes targeted by all
217 antibiotic resistance genes found in *A. lwoffii* and *A. baumannii* whole genome sequences. Only 40
218 different ARGs were found for *A. lwoffii* whereas 333 different ARGs were found across *A. baumannii,* but
219 this is likely explained by the different dataset sizes of either species. EP - efflux pump associated genes
220

221 ***A. lwoffii* and *A. baumannii* possess similar genomic signatures of horizontal gene transfer,**
222 **but *A. lwoffii* contains more DNA defence systems**
223 The greater antibiotic resistance levels of *A. baumannii* are seemingly explained by the fact
224 that this species harbours significantly more ARGs than *A. lwoffii*. However, both species
225 inhabit similar niches, cause similar types of infection, and therefore are expected to have
226 been exposed to similar antibiotics. Variation in rates of horizontal gene transfer into and
227 within each species might explain the difference in the numbers of ARGs they carry. To
228 investigate this, the presence of prophage and plasmid-associated sequences, type IV pili
229 genes for natural transformation and the presence of DNA defence systems, which would
230 limit the acquisition of foreign DNA, were searched for in the whole genome sequences.
231

232 To determine whether *A. baumannii* and *A. lwoffii* harbour different numbers or types of
233 plasmids, ABRicate was used to screen for plasmid replicons from an *Acinetobacter*
234 replication initiation (*rep*) gene database (23). An average of 4 and 2 *rep* genes were found
235 per *A. lwoffii* and *A. baumannii* genome, respectively. A random permutation and Welch's T
236 test revealed that *A. lwoffii* contained significantly more *rep* genes than *A. baumannii* (p
237 <0.0001), suggesting that *A. lwoffii* harbours more plasmids, Fig. 2a.
238
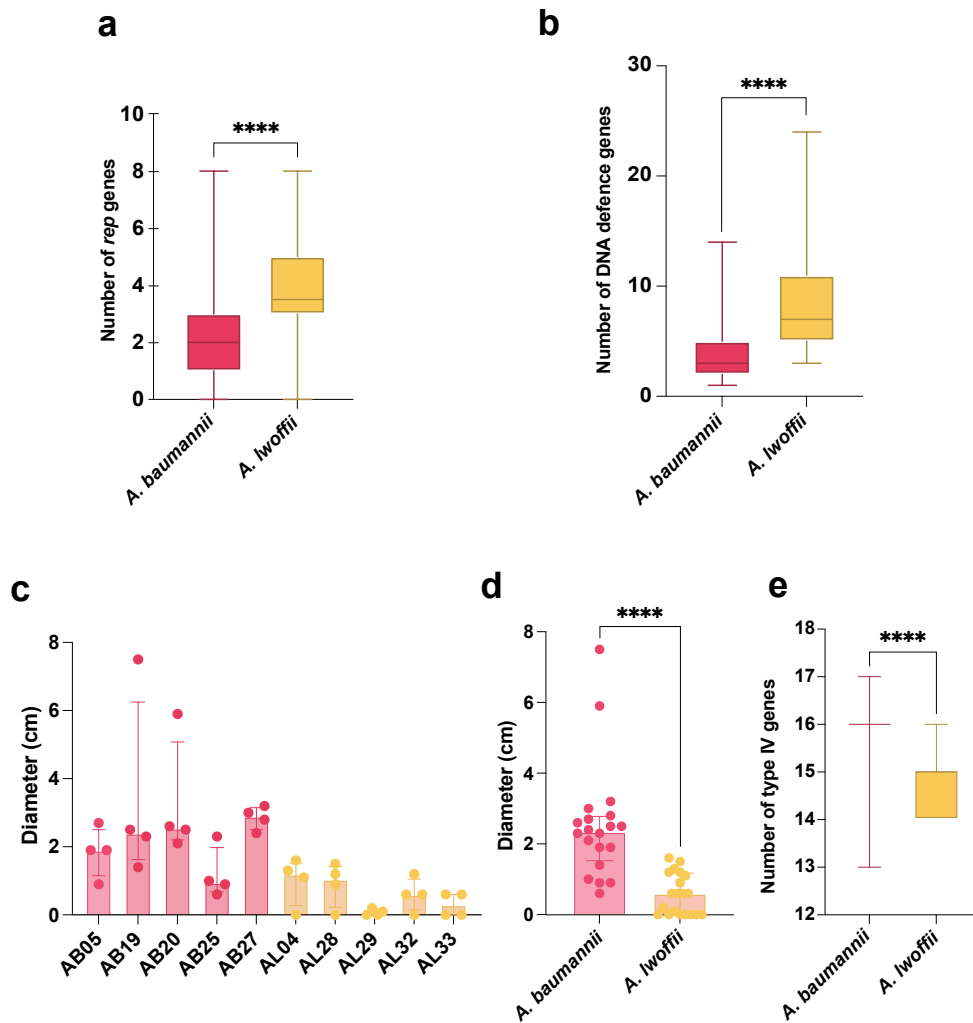
Fig. 2 Signatures of foreign DNA acquisition. a - Number of *rep* genes found per whole genome sequence from either species, b – number of DNA defence system genes per sequence. *A. baumannii* in pink n= 4,809, *A. lwoffii* in yellow n=38. c- the twitching motility of individual strains, d- combined values of all strains tested per species, and e- the number of type IV pili associated genes found in the whole genome sequences. A random permutation and Welch's T-test was used to compare the mean number of *rep,* DNA defence system and type IV associated genes in comparable datasets of *A. baumannii* and *A. lwoffii*, *A. baumannii* genomes encode more genes than *A. lwoffii* (in all instances p <0.0001).

Acinetobacter rep genes are classified broadly according to the protein family they encode (Rep_1, Rep_3 or RepPriCT_1) and specifically by homology (>95% nucleotide identity cut-off) to a collection of reference *rep* sequences (23). All *A. lwoffii* rep genes detected here belonged to the Rep_3 (R3) group. However, since the *rep* database was constructed primarily for the purpose of typing plasmids in *A. baumannii,* there were inconsistencies when comparing the *rep* genes identified by ABRicate and the number of circular plasmid sequences in complete *A. lwoffii* genomes. ABRicate detected fewer *rep* genes (n=34) than there were plasmids (n=64) in the complete genomes (supplementary S5). Whilst it is possible that some plasmids did not contain a recognisable *rep* gene, as has been reported for *A. baumannii* plasmids (23), this was unlikely to be the case for all instances here. Therefore, the NCBI annotations for all plasmids in complete *A. lwoffii* genomes were screened for ORFs labelled "rep", and a further six genes not represented in the database were found, five encoding Rep_3 proteins -

262 CP032104 1 (pALWEK1.11), CP080579 1 (pALWVS1.3), CP072552.1 (pH7-68), CP080580 1
263 (pALWVS1.4), CP080643 1 (pALWEK1.16) and one encoding Rep_1 (CP080641; pALWEK1.14).
264 In a phylogenetic tree, these genes clustered independently of previously known *rep* genes
265 (supplementary S6). With these considered, all but one *A. lwoffii rep* genes clustered in R3,
266 supporting the idea that *A. lwoffii* almost exclusively maintains R3-type plasmids.

268 The most common *rep* types in *A. lwoffii* were R3-T25/R3-T45, which were found in a total of
269 92% of genomes. R3-T25 and R3-T45 are 94.71% identical at the nucleotide level and
270 therefore, although classed as different *rep* types using a 95% cut-off value, are very closely
271 related. Therefore, we propose that R3-T25/R3-T45 replicons represent a native *A. lwoffii*
272 plasmid family, found in almost all complete genome sequences of this species examined
273 here. In contrast, R3-T25/R3-T45 replicons were only found in 0.4% of *A. baumannii* genomes.
274 For *A. baumannii,* 38% of sequences contained R2-T1 and 37% encoded RP-T1 *rep* types. In
275 total, *A. baumannii* had 82 distinct *rep* types, including from RP, R1, R2 and R3 groups. A full
276 list of *rep* genes highlighted in both species' can be found in supplementary S7.

278 In addition to ARGs, occasionally, plasmids may also carry genes for RND efflux pumps, which
279 can export a wide range of structurally diverse compounds, including antibiotics (12), and can
280 act as important mechanisms for antibiotic resistance. RND determinants have been seen in
281 plasmids in *A. baumannii,* for example pDETAB2 from a Chinese ICU patient isolate (32), and
282 more recently in *A. lwoffii*, where AL_065, which was isolated from a hospital bed rail in
283 Pakistan, harboured a plasmid encoding an RND transporter and periplasmic adaptor protein
284 (33). This plasmid (CP078046.1, *rep* type R3-T25) is also found in *A. baumannii* and has the
285 potential to disseminate RND efflux genes across *A. lwoffii* more broadly. The RND pump is
286 closest in homology to AdeB (31) and may therefore represent the acquisition of an
287 additional, adaptive RND pump, reducing the susceptibility of this strain to structurally
288 different substrates than those exported by its native RND system: AdeIJK (1).

290 To determine if the relative lack of ARGs in *A. lwoffii* could also be related to other
291 mechanisms of HGT, we searched for the presence of prophage DNA within genomes of both
292 species. Both *A. lwoffii* and *A. baumannii* had prophage DNA within their genomes, as
293 determined by PHASTER (supplementary S8). Therefore, both species have been previously
294 infected by phage and have the capacity to acquire novel DNA, such as ARGs, introduced by
295 phages.

297 The number of DNA defence systems across the two species was determined as this could
298 impact their acquisition and maintenance of foreign DNA. Using DefenseFinder *A. lwoffii*
299 genomes were found to encode between 3 and 24 defence systems per genome which was
300 significantly more than *A. baumannii* which had between 1 and 14 (p=<0.0001) (Fig. 2b). The
301 types of defence systems present also differed. *A. lwoffii* encoded mostly type I and IV
302 restriction modification systems, which cleave unmethylated DNA whereas *A. baumannii*
303 encodes more PsyrTA toxin antitoxin systems and antiphage systems e.g. SspBCDE
304 (supplementary S9).

306 *Acinetobacter* species can display twitching motility in laboratory conditions, which aids the
307 natural transformation of DNA from the extracellular environment into the cell (22).
308 Therefore, the ability of *A. lwoffii* and *A. baumannii* to twitch was measured. While there was

309 strain variation in sub surface twitching motility, generally *A. lwoffii* twitched less (average of
310 0.6 cm) than *A. baumannii* (average of 2.5 cm) at 37°C, Fig. S2 (c,d), suggesting that *A. lwoffii*
311 may be less naturally competent than *A. baumannii*.
312
313 Natural transformation uses type IV pili genes and therefore we also looked for the presence
314 of genes associated with type IV pili in both species (Fig. 2e). There were significantly more
315 type IV associated genes found in *A. baumannii* genomes compared to *A. lwoffii* genomes
316 (p<0.001), supplementary S10.
317
318 ***A. baumannii* readily evolved resistance to meropenem, ciprofloxacin and gentamicin but**
319 ***A. lwoffii* only evolved resistance to ciprofloxacin**
320 Since *A. lwoffii* has remained susceptible to antibiotics, we sought to determine whether it
321 can evolve resistance to clinically relevant antibiotics *in vitro*. For context, we also included *A.*
322 *baumannii*, which is well known to evolve drug resistance rapidly. To this end, selection
323 experiments were set up, where susceptible strains were grown in the presence of increasing
324 concentrations of meropenem, ciprofloxacin or gentamicin. After 7 days, whole genome
325 sequencing was performed to characterise any genomic changes compared to the ancestral
326 strain (supplementary S11).
327
328 *A. baumannii* (AB18) mutants selected in the presence of meropenem had meropenem MICs
329 2-3-fold above that of the parent strain MIC, from 1 to 2-4 μg/mL (supplementary S11). There
330 were also MIC increases for ampicillin (4-5-fold), ciprofloxacin (3-fold) and tetracycline (3-
331 fold) with some mutants also being less susceptible to moxifloxacin (2-3-fold) and
332 erythromycin (2-3-fold), Fig. 3. It was noted that fewer *A. lwoffii* (AL28) colonies were selected
333 for, however when MIC testing the mutants, the increase was also 3-fold above the ancestral
334 MIC from 0.015 to 0.06 μg/mL. There was no significant MIC change for the other antibiotics
335 tested.
336
337 Five mutants from AL28 and AB18 were subject to whole genome sequencing and their
338 sequences were compared to the ancestral strain and parental strains which had been
339 passaged in the same experiment in nutrient broth only. Despite *A. lwoffii* being able to grow
340 at the final concentration of meropenem used in the evolution experiment no canonical
341 resistance mutations were seen. In fact, no SNPs were found in the mutants, even though the
342 strains passaged in nutrient broth alone encoded some SNPs. However, for *A. baumanii*
343 (AB18) all five sequenced strains had SNPs in the RND efflux transporter encoding gene *adeJ*
344 and in the gene that encodes the repressor protein for this system - *adeN*. Three of the *adeJ*
345 mutations were within the distal binding pocket of the pump, where beta-lactams bind (1).
346 Additionally, AB18 mutant 5 had mutations in genes encoding penicillin binding proteins 2
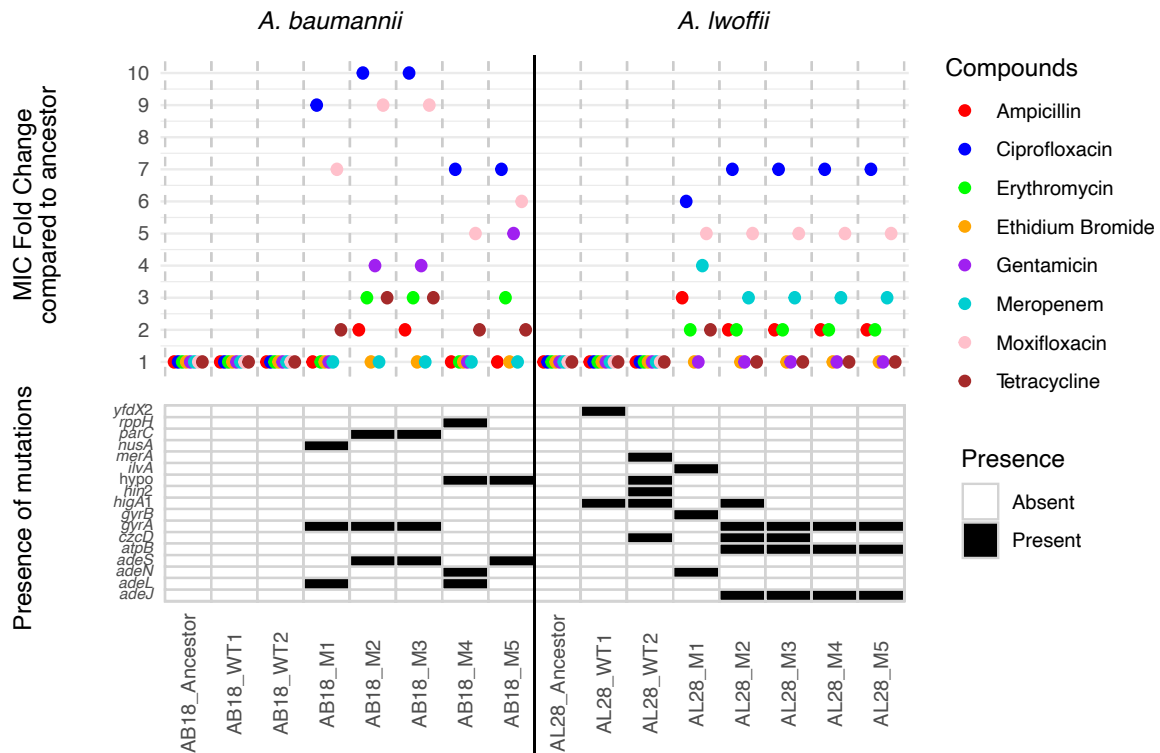347 and 3, known to be involved in meropenem resistance (34).
348
349
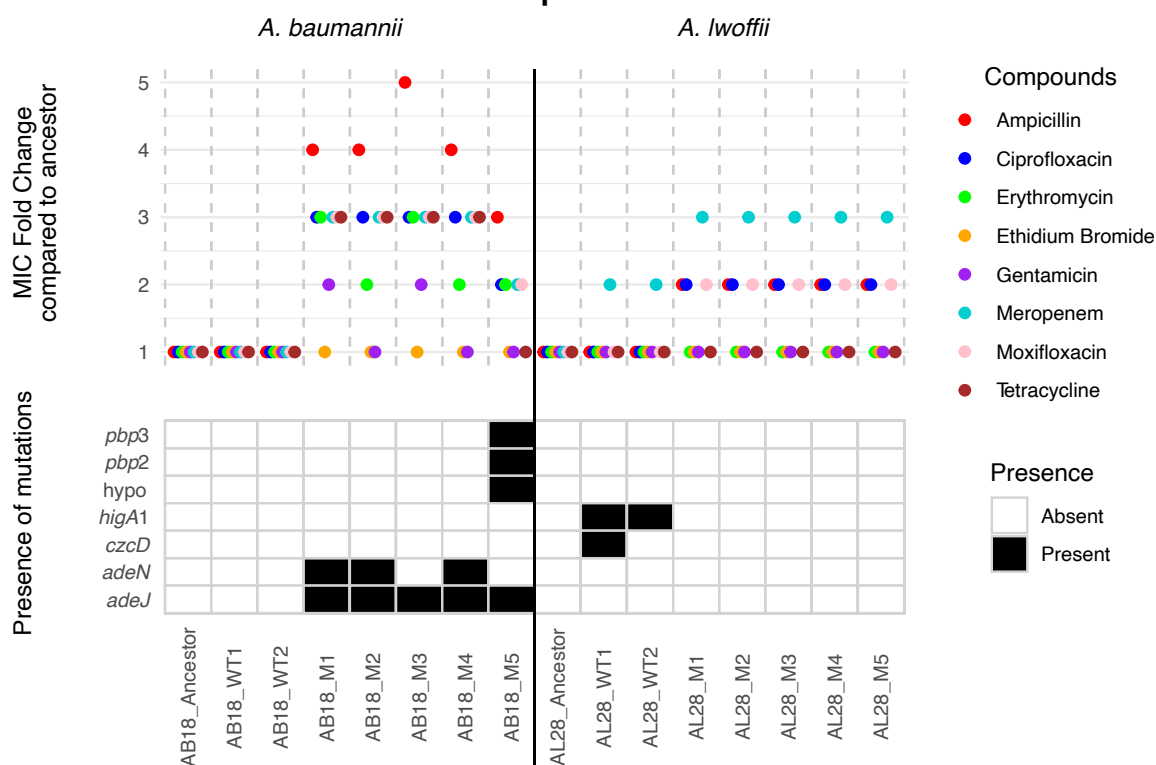
Fig. 3 MIC fold change results and SNP presence for strains evolved to grow in increasing concentrations of meropenem. "Ancestor" and "WT" (broth-only) controls are compared to individual "M" (mutant) isolates from the terminal passage. AB18 - *A. baumannii* and AL28 - *A. lwoffii*. An MIC fold change of 1 means the strain is as susceptible or more susceptible to the drug compared with the ancestor.

For ciprofloxacin both *A. baumannii* and *A. lwoffii* cultures evolved resistance to above the EUCAST breakpoint. In AB18 large MIC changes, between 9 and 10-fold higher than the ancestral strain, were seen for ciprofloxacin and moxifloxacin. Additionally, MIC increases were also observed for gentamicin (4-5-fold) and erythromycin (3-fold) in some mutants (AB18 M2, M3, M5) and the tetracycline MIC was also elevated (3-fold) in AB18 M2 and M3. Mutants selected in the presence of increasing concentrations of ciprofloxacin had mutations in both the target of the drug (*gyrA/gyrB/parC*) and RND efflux systems (*ade* pumps).

For *A. lwoffii*, in contrast to the results seen with meropenem, target site and efflux SNPs were seen in the AL28 mutants. It is also worth noting the AL28 WT strains also harboured polymorphisms, despite being passaged in nutrient broth only. SNPs were found in genes such as *higA*1, encoding an antitoxin protein, and *yfdX*2, encoding a heat resistance protein. AL28 mutants had SNPs in *adeJ, adeN, atpB, gyrA* and *gyrB*. Presumably, the combination of SNPs in efflux- related genes and target-site genes contributed to the reduced susceptibility of the mutants to ciprofloxacin, moxifloxacin and also protected against meropenem, Fig. 4.
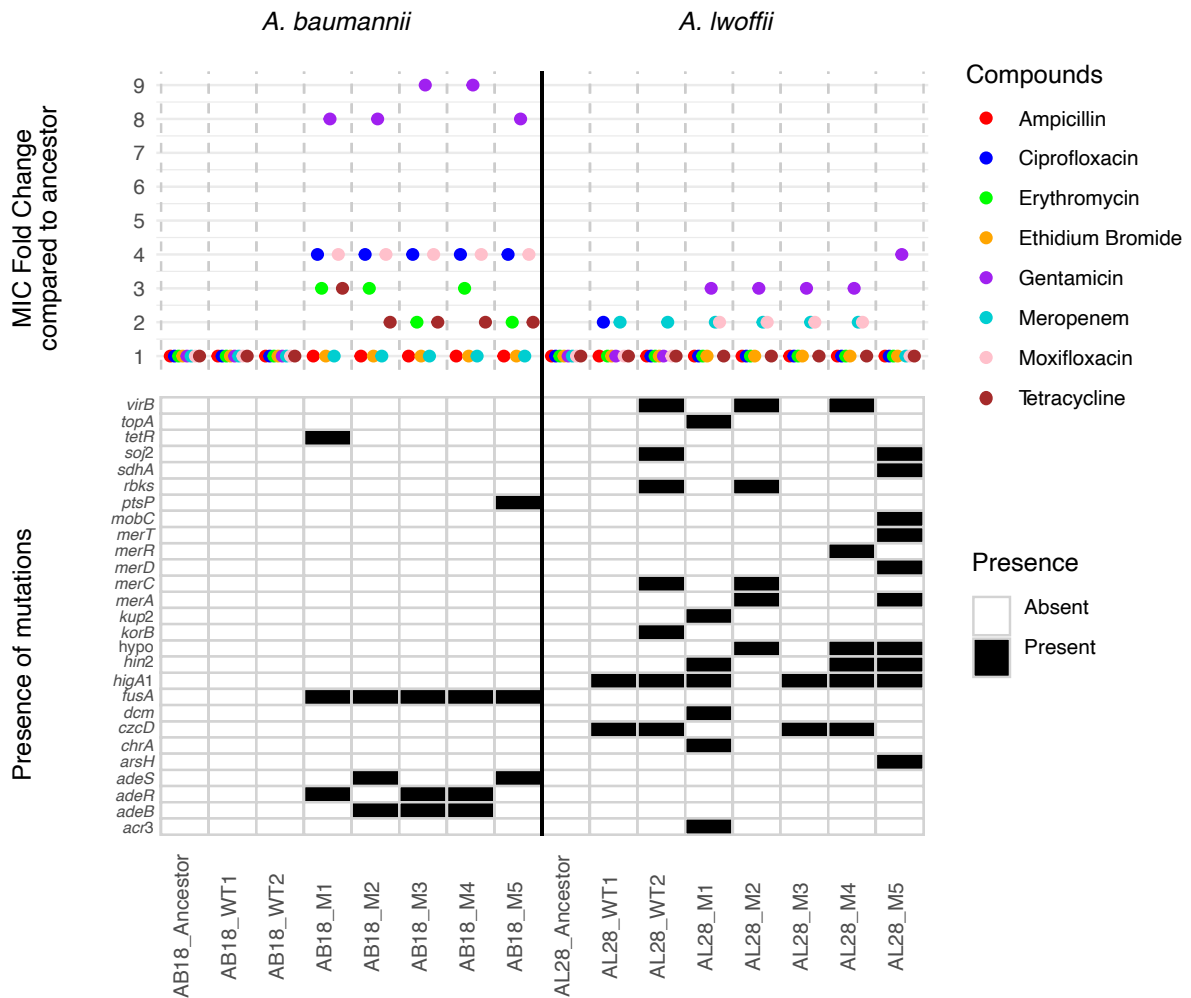
Fig. 4 MIC fold change results and SNP presence for strains evolved to grow in increasing concentrations of ciprofloxacin. "Ancestor" and "WT" (broth-only) controls are compared to individual "M" (mutant) isolates from the terminal passage. AB18 - *A. baumannii* and AL28 - *A. lwoffii*. An MIC fold change of 1 means the strain is as susceptible or more susceptible to the drug compared with the ancestor.

Since *A. lwoffii* seemed to be capable of evolving drug resistance mutations to ciprofloxacin but not meropenem a third experiment was conducted. Here, gentamicin was chosen which is also used clinically to treat *Acinetobacter* infections. All AB18 mutants had elevated MICs to gentamicin (8 or 9-fold above ancestral strain MIC), taking them from clinically susceptible to resistant (> 4 μg/mL), Fig. 5. These mutants also displayed a reduced susceptibility to ciprofloxacin and moxifloxacin and some of the AB18 mutants (1, 2 and 4) also showed a reduced susceptibility to erythromycin and tetracycline too. The wild-type strains grown in broth did not encode any SNPs, whereas the mutant strains had SNPs in *adeB, adeR, adeS, fusA, ptsP* and *tetR*.

As with meropenem, the *A. lwoffii* strain tested did not exhibit drug resistance to gentamicin or other drugs tested. However, during this experiment many SNPs were selected for in both the nutrient broth only conditions (WT1 and WT2) and gentamicin conditions (M1-5). Mutations found only in the AL28 cells grown in gentamicin included SNPs in *acr*3 and *arsH* (arsenic resistance), *chrA* (chromate resistance) and *merA, merD merR* and *merT* (mercuric transport proteins). Therefore, there was both conservative MIC differences and genomic evidence of a stress response, particularly in metal-tolerance genes.

Fig. 5 MIC fold change results and SNP presence for strains evolved to grow in increasing concentrations of gentamicin. "Ancestor" and "WT" (broth-only) controls are compared to individual "M" (mutant) isolates from the terminal passage. AB18 - *A. baumannii* and AL28 - *A. lwoffii*. An MIC fold change of 1 means the strain is as susceptible or more susceptible to the drug compared with the ancestor.

In summary, *A. baumannii* AB18 was able to rapidly evolve resistance to three clinically relevant antibiotics, which provided not only elevated MICs to that antibiotic but also to drugs from other classes. Furthermore, AB18 went from clinically susceptible to resistant, as defined by EUCAST breakpoints, in each instance. However, for *A. lwoffii*, clinical resistance was only seen for ciprofloxacin. These results show that *A. lwoffii* has a more limited capacity to evolve resistance to antibiotics and due to the diversity of efflux-related mutations in *A. baumannii* this may be due to the lack of RND systems in *A. lwoffii*.

A. lwoffii forms less biofilm, and the biofilm is more susceptible to antibiotics and biocides than those formed by A. baumannii.

Antibiotic susceptibility is known to be decreased when bacteria exist within a biofilm and *Acinetobacter* often forms biofilm to aid survival in the clinical environment (35). Therefore, the biofilm forming capacity and susceptibility of biofilm to antibiotics was determined. In static conditions, *A. baumannii* strains formed significantly more biofilm on average than the

416 *A. lwoffii* strains (p<0.001), median OD$_{600}$ values of 3.39 and 0.53 respectively, supplementary
417 S12 (a, b). However, when biofilm was formed under laminar flow conditions there was no
418 significant difference in the amount of biofilm formed between the two species,
419 supplementary S12c.
420
421 When the genomes were searched, for genes previously associated with biofilm formation
422 (24), *A. lwoffii* sequences were found to have a mean of 1 gene per sequence whereas *A.*
423 *baumannii* had a mean of 8 genes per genome sequence (supplementary S12d, S13).
424 However, as this database was created using genes from *A. baumannii*, biofilm-associated
425 genes exclusive to or uncharacterised in *A. lwoffii* would not have been found using this
426 approach.
427
428 Given that a biofilm lifestyle is associated with decreased susceptibility to antibiotics the MIC
429 and minimum biofilm eradication concentration (MBEC) was determined for representatives
430 of both species (Table 2). For both species the MBEC values were generally higher than the
431 MIC values, for example for AB20 the cefotaxime MBEC was 10-fold higher than the MIC.
432 However, the effect was less evident in *A. lwoffii* (AL04), where there were instances where
433 the MBEC and MIC values did not significantly change (chlorhexidine, meropenem and
434 triclosan). Furthermore, in general the *A. lwoffii* (AL04) MBEC values were lower than those
435 of *A. baumannii* (AB20). Therefore, whilst the biofilms formed by both strains were less
436 susceptible to antibiotics and biocides, the biofilm formed by *A. baumannii* (AB20) afforded
437 greater protection than in *A. lwoffii*.
438
439 **Table 2** Minimum biofilm eradication concentrations (MBEC) and minimum broth inhibitory
440 concentrations (MIC) of antibiotics and biocides in *A. lwoffii* (AL04) and *A. baumannii* (AB20).

	AL04		AB20	
	MBEC	MIC	MBEC	MIC
Cefotaxime (µg/mL)	256	2	8192	16
Chlorhexidine (%)	0.0017	0.0008	<1	0.0035
Ciprofloxacin (µg/mL)	16	0.06	128	2
Meropenem (µg/mL)	0.06	0.03	<128	0.25
Oxacillin (µg/mL)	8	16	<4096	512
Tetracycline (µg/mL)	256	0.5	1024	2
Triclosan (µg/mL)	0.5	0.5	<128	1
Rifampicin (µg/mL)	8	0.5	64	4

441
442 ***A. lwoffii* has a longer cell morphology than *A. baumannii***
443 Thus far it is clear that *A. lwoffii* is more susceptible to antibiotics than *A. baumannii*, in both
444 static and biofilm conditions and this is likely due to a reduced ability to evolve and acquire
445 resistance, which may be underpinned by the presence of more DNA defence systems and
446 fewer RND efflux pumps. Given the lack of research into *A. lwoffii*, the basic biology of the
447 two species under laboratory conditions was assessed.
448
449 To determine whether there were any morphological differences between these two species,
450 two strains of *A. baumannii* (AB05, AB18) and two strains of *A. lwoffii* (AL04, AL28) were

451 imaged using scanning electron microscopy (SEM). *A. lwoffii* had significantly longer cells than
452 *A. baumannii,* (n=100 cell measurements per strain) Fig. 6, supplementary S14.
453

Average cell length
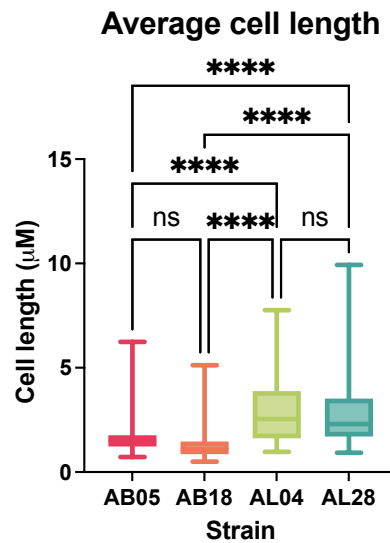
454
455
456 **Fig. 6** Average cell length (μM) of *A. baumannii* (AB) and *A. lwoffii* (AL) strains imaged by the Apreo 2
457 Scanning Electron Microscope. *A. lwoffii* had statistically longer cells than *A. baumannii* strains
458 (*****p=<0.0001, one-way ANOVA with Tukey's multiple comparisons). The whiskers on the box plot
459 show minimum and maximum values obtained.
460

A. baumannii grows more readily in both LB and human serum than *A. lwoffii*

462 Additionally, the growth of both species was compared at 37°C, 30°C and 25°C. In LB *A. lwoffii*
463 grew to a lower final density than *A. baumannii* at all temperatures. Growing at cooler
464 temperatures generally increased the length of the lag phase. The mean generation times
465 (supplementary S15) were generally faster at 30°C for *A. lwoffii* while *A. baumannii* grew
466 fastest at 37°C. While *A. lwoffii* grew to a lower final OD than *A. baumannii* (supplementary
467 S16) the generation times of AL28, AL32 and AL33 grew at comparable rates to the *A.*
468 *baumannii* strains.
469

470 Due to the capacity of both species to cause bacteraemia in humans, we also sought to
471 understand how well both species survive and grow in human serum. Growth was compared
472 in human serum with and without complement proteins (normal human serum (NHS) and
473 heat inactivated serum (HIS), respectively); both species grew more slowly in serum than LB
474 (supplementary S17 and S18). Of the two *A. lwoffii* strains tested, AL04 had a prolonged lag
475 but did grow in both HIS and NHS, although growth rate was better in HIS. AL28 did not grow
476 in serum and formed clumps, making OD measurements problematic. *A. baumannii* AB05 and
477 AB18 grew as well in normal serum and as they did in HIS. AB18 grew significantly (p=0.0098)
478 better than AB05 in HIS. All other conditions were not significantly different.
479

480 Survival in human serum was also measured, to determine whether, although not actively
481 growing, strains could remain viable in the presence of serum. All strains, except AL28, could
482 survive in NHS and by 24 hours CFU/mL was similar in both serum and the LB control, Fig. 7a.
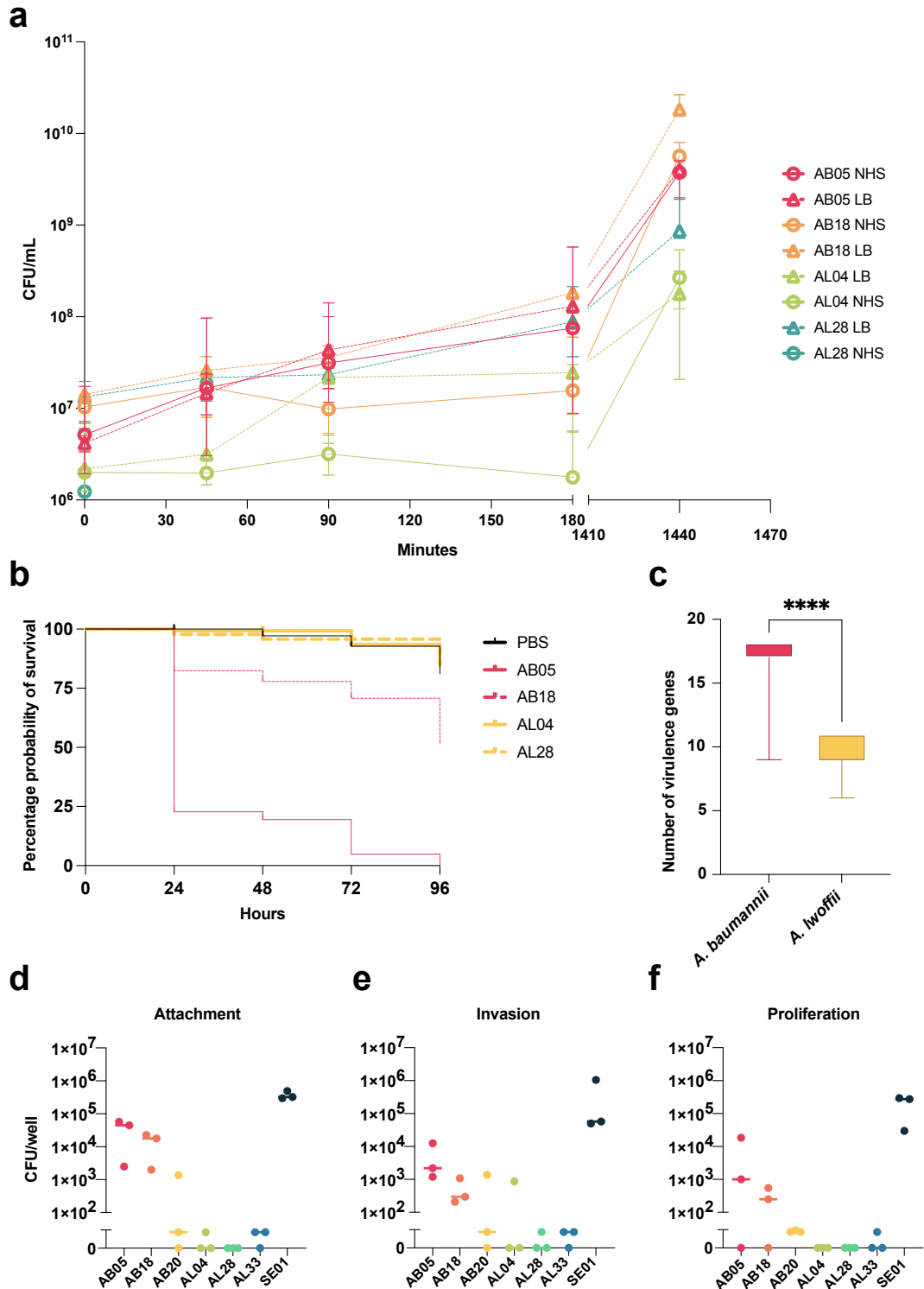
483
484 **Fig. 7** a- the survival of *A. baumannii* (AB) and *A. lwoffii* (AL) strains in both LB (dashed line) and normal
485 human serum (continuous line) over 24 hours. b- Survival of *Galleria mellonella* after inoculation with
486 either *A. baumannii* (AB, pink) or *A. lwoffii* (AL, yellow). A PBS injury control is included in black. c- the
487 number of virulence associated genes found in *A. baumannii* (pink) and *A. lwoffii* (yellow) whole genome
488 sequences. A random permutation and Welch's T test was used and shows that *A. baumannii* encodes
489 significantly more virulence genes than *A. lwoffii*. Attachment (d), invasion (e) and proliferation (f) of both
490 species in human THP-1 macrophages was measured. SE01 is a positive control of *Salmonella enterica*
491 Typhimurium. AB = *A. baumannii* AL = *A. lwoffii.* Comparative statistics (one-way ANOVA) were performed
492 but no conditions were significantly different.

493
494 A synthetic wound model (36) also showed that *A. lwoffii* strains did not grow as well as *A.*
495 *baumannii* strains. This supports the fact that *A. lwoffii* survived poorly in the presence of
496 human serum as AL04 and AL28 did not grow, supplementary S19.
497
498 **A. baumannii is more virulent than A. lwoffii in vivo and in vitro**
499 We also sought to determine whether there was a difference in the *in vivo* virulence capacity
500 of the two species and chose to use the well-characterised infection model organism *Galleria*
501 *mellonella,* which has an innate immune system (30). When *G. mellonella* larvae were
502 infected with $1x10^6$ *A. baumannii* or *A. lwoffii* cells, more larvae were killed when infected
503 with *A. baumannii* (AB05 and AB18) than *A. lwoffii* (AL04 and AL28) which correlates with
504 what has been seen previously (30). By 48 hours the probability of larvae survival was <25%
505 for AB05 infection, whereas it was >95% for AL28. Of the *A. baumannii* strains, AB05 was
506 significantly more virulent than AB18 in this model, Fig. 7b (p<0.0001, Log-rank test).
507
508 Since *A. baumannii* was more virulent *in vivo* than *A. lwoffii*, we also probed the whole
509 genome sequences for the presence of virulence genes. *A. baumannii* genomes encoded
510 significantly more virulence genes than *A. lwoffii* genomes, p<0.0001, (Fig. 7c, supplementary
511 S20) when using a random permutation and T test to compare two equally sized sample sets.
512
513 Finally, to determine virulence *in vitro*, strains were incubated with a human macrophage cell
514 line, THP-1. *A. baumannii* strains (AB05 and AB18) were able to attach to and subsequently
515 invade THP-1 cells Fig. 7 (d and e). However, after six hours proliferation was also measured
516 and there was no difference in the number of CFUs between invasion and proliferation
517 suggesting *A. baumannii* was not actively growing within the cells but could survive at least
518 for the period of the assay, Fig. 8f. In contrast, neither of the *A. lwoffii* strains tested could
519 attach to or invade human macrophage cells *in vitro*.
520
521 **Discussion:**
522 The emerging pathogen, *A. lwoffii* is the leading cause of *Acinetobacter*-derived blood stream
523 infections in England and Wales, followed by the extensively studied *A. baumannii* (4).
524 However, *A. baumannii* has developed widespread multidrug resistance while *A. lwoffii* has
525 remained sensitive to almost all antibiotics. Whilst research into *A. baumannii* is increasing
526 and more is known about its antibiotic resistance there remains a knowledge gap in
527 understanding the emerging opportunistic pathogen *A. lwoffii*. This work aimed to explore
528 differences in the two species in terms of their antibiotic susceptibility, infectivity, and basic
529 biology. We have shown that *A. lwoffii* is more susceptible to drugs used to treat
530 *Acinetobacter* infections than *A. baumannii*, is less virulent and does not evolve drug
531 resistance to the same degree as *A. baumannii*.
532
533 This work confirmed previous data suggesting *A. lwoffii* is susceptible to antibiotics while *A.*
534 *baumannii* is commonly multidrug resistant (4) and showed the difference in phenotype is
535 caused by *A. lwoffii* encoding fewer resistance genes than *A. baumannii*. Both species are
536 found in similar environments such as on the human body, although *A. baumannii* is not
537 considered to be part of a healthy skin microbiome (5, 37). As they are both found within the
538 hospital environment, it is peculiar that resistance (either by mutation or the horizontal
539 acquisition of ARGs) has not been commonly selected for in *A. lwoffii*.

540

541 The lack of ARGs in *A. lwoffii* may be due, at least in part, to the presence of DNA defence
542 systems that are absent in *A. baumannii,* such as a greater number of restriction modification
543 systems. The presence of more DNA defence systems in *A. lwoffii* suggests that this species is
544 more stringent about the DNA it maintains (38).

545

546 In addition to fewer ARGs, *A. lwoffii* also less readily evolved resistance to three clinically
547 relevant drugs compared to *A. baumannii*. Drug resistance mutations often occur in the drug's
548 target: penicillin binding proteins for meropenem (34), DNA gyrase for ciprofloxacin (39–41)
549 and the ribosome for gentamicin (42). This was the case for *A. baumannii* here. In the one
550 instance where *A. lwoffii* evolved resistance, to ciprofloxacin, drug target mutations were also
551 observed. Ciprofloxacin mutations often occur in the quinolone resistance determining
552 regions (QRDR) of GyrA, GyrB and ParC (41). The *A. baumannii* mutations in *gyrA* were in the
553 QRDR (amino acids 65-104) but *A. baumannii* mutations in *parC* and *A. lwoffii* in *gyrB,*
554 however, were not within the QRDRs.

555

556 Additionally, mutations were captured in RND efflux pumps that export the compounds used
557 for selection. For example, *A. baumannii* meropenem mutants had *adeJ* mutations and beta-
558 lactams bind to the distal pocket of AdeJ (42, 43). Fluoroquinolones can be exported by all
559 three Ade pumps in *A. baumannii* (37), which explains why mutations in all three Ade systems
560 were seen, including mutations that affected the regulators of these systems. Gentamicin is
561 exported by AdeB and can bind to both the proximal and distal binding pockets but Y77, T91,
562 and S134 are thought to be essential for gentamicin binding to the proximal pocket of AdeB
563 (44). Given the proximity of the *A. baumannii* AdeB mutations (amino acids 97 and 136) in
564 this study to those reported in the literature (44), it is likely that these mutations led to
565 increased gentamicin export via AdeB. Mutations in AdeRS have been reported to increase
566 AdeABC expression, for example A91V in AdeR and A94V in AdeS (45). This study also
567 captured the A91V SNP in AdeR, which sits in the signal receiver domain, as well as other
568 mutations in AdeRS, indicating that AdeRS may be being modulated to increase AdeABC
569 expression and the extrusion of gentamicin.

570

571 The mutant evolution experiments clearly show that *A. lwoffii* has a reduced capacity to
572 evolve resistance to antibiotics compared to *A. baumannii,* where it only evolved resistance
573 to ciprofloxacin. This could be because *A. lwoffii* only encodes one tripartite RND system
574 (AdeIJK) (1). RND efflux pumps have an underpinning role in the development of resistance
575 via other molecular mechanisms (42). For example, in other species of Gram-negative
576 bacteria deletion of efflux pumps reduces the mutation selection frequency (42, 46). In
577 addition, mutations within efflux pumps often occur first evolutionarily and allow for the
578 development of more canonical drug target mutations, which may have been the case in this
579 study (47, 48). The reduced efflux capacity of *A. lwoffii* could therefore limit the selection of
580 drug resistance mutations. This is further supported by the fact that in *A. baumannii*, drug
581 resistance mutations were found across all three tripartite systems, indicating their important
582 role in resistance evolution. Another potential mechanism for the lack of resistance
583 development could be more stringent DNA repair mechanisms in *A. lwoffii,* for example
584 mismatch repair to inhibit the recombination of non-homologous DNA (49).

585
586

587 When looking at infection-related phenotypes, *A. baumannii* was more virulent than *A.*
588 *lwoffii*. It was already known that certain *A. baumannii* strains could infect macrophages and
589 persist within their vacuoles, but this was the first time this experiment had been done using
590 *A. lwoffii,* where none of the strains tested could persist within macrophages (31). This could
591 indicate that it is easier to clear *A. lwoffii* infections.
592
593 In summary *A. lwoffii* is more susceptible to antibiotics than *A. baumannii* due to a lack of
594 acquired and evolved resistance. Ultimately, an open question remains surrounding why *A.*
595 *lwoffii* does not seem to be developing drug resistance in the clinic and more work is
596 needed to elucidate if this results from a lack of efflux systems and/or more stringent DNA
597 repair and defence, or other factors. Whilst the widespread antibiotic susceptibility of *A.*
598 *lwoffii* allows for successful clinical outcomes, there are sporadic cases of drug resistant *A.*
599 *lwoffii,* highlighting the possibility that drug resistance could emerge (9, 50). It is, therefore,
600 important to fully chart the development of this emerging pathogen to limit the
601 development of drug resistance.
602

626
627

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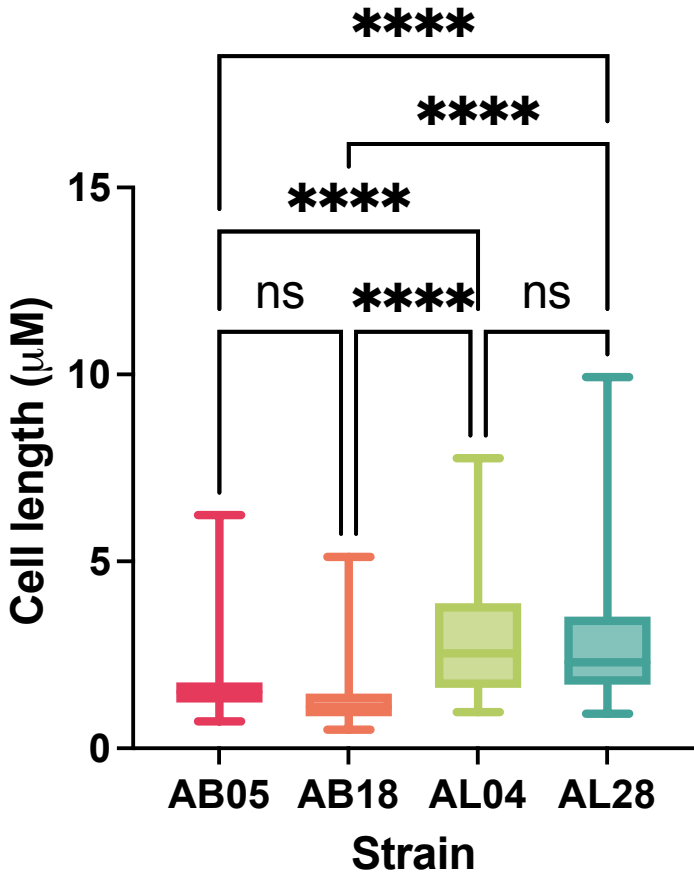
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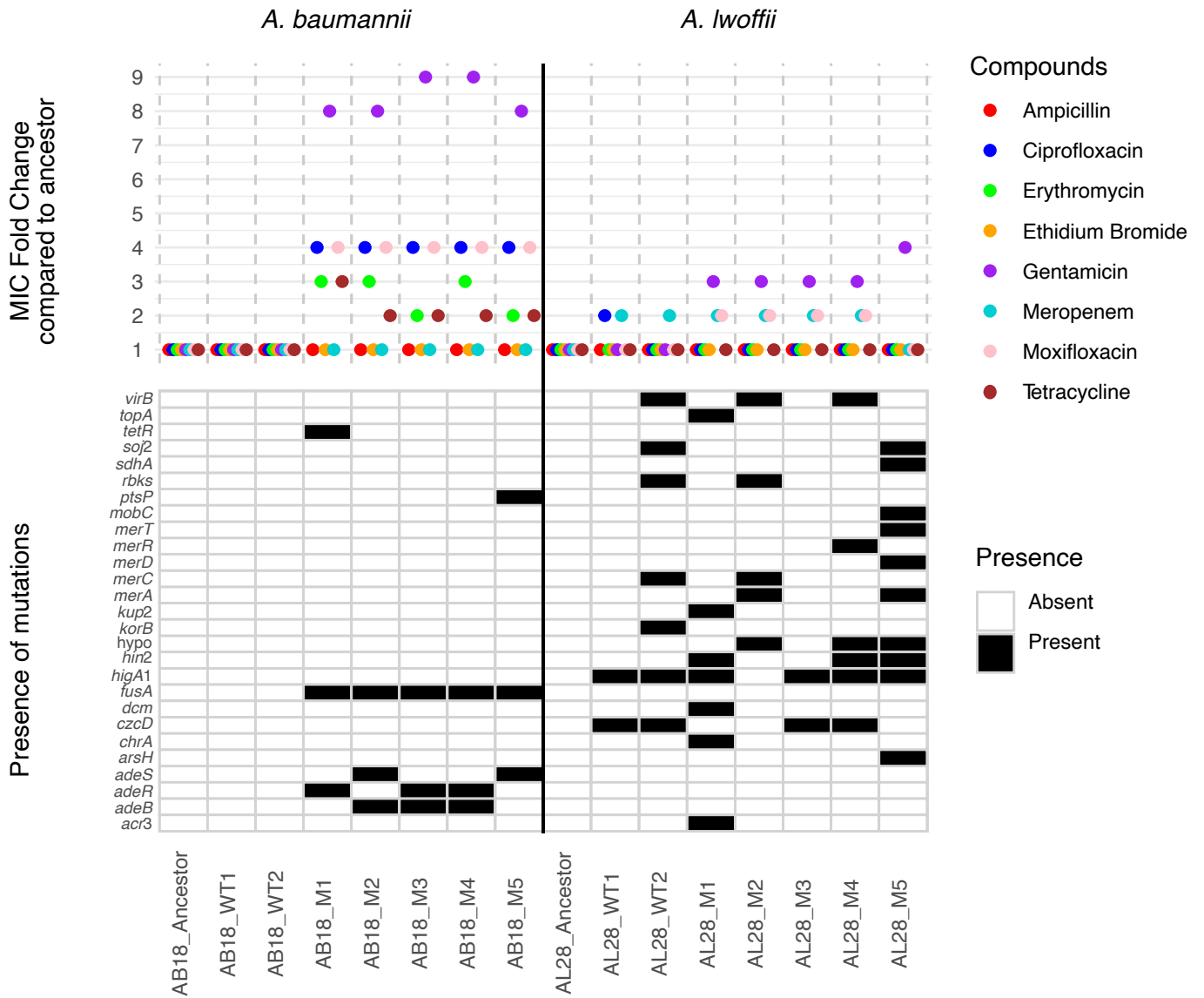729   addition to cellular defense. Microbiology and molecular biology reviews 77:53–72.

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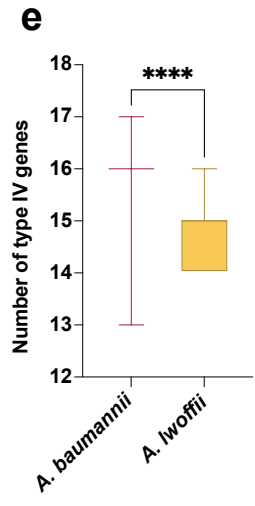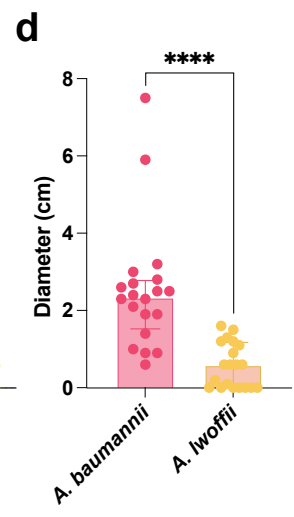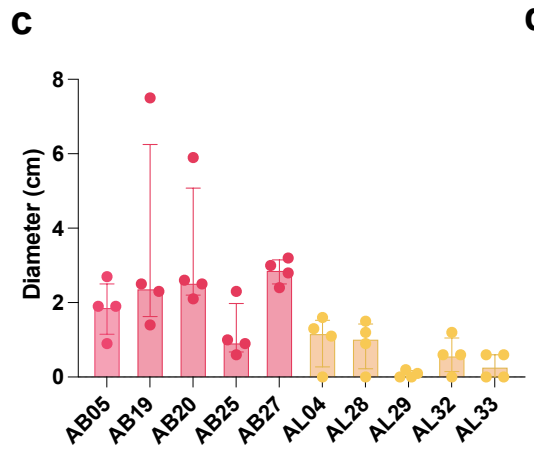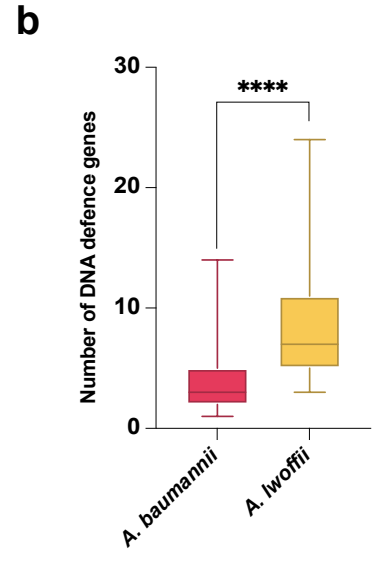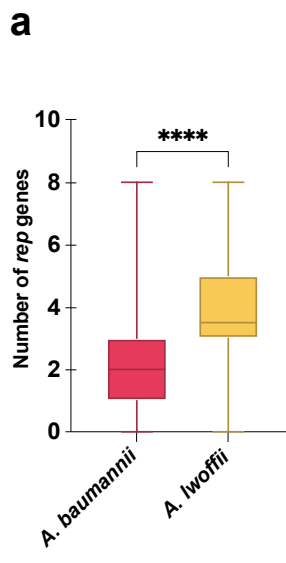749   *Acinetobacter baumannii* multidrug efflux pump. MBio 10:10–1128.

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Strains evolved to grow in the presence of gentamicin

a

b

Legend (panel b):
- β lactam
- Efflux pump
- Aminoglycoside
- Efflux regulator
- Phenicol
- Macrolide
- Sulfonamide
- Bleomycin
- Rifamycin
- Peptide
- Tetracycline
- Trimethoprim
- Quinolone
- Fosfomycin
- Streptogramin
- Lincosamide
- Porin
- Nucleoside

Strains evolved to grow in the presence of ciprofloxacin

Strains evolved to grow in the presence of meropenem

a

b

c

d   Attachment

e   Invasion

f   Proliferation