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1 **Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient,**
2 **where resistance developed during tigecycline therapy**

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16 Running title: Genome sequencing of *A. baumannii* from a patient treated with tigecycline

17 Keywords: OXA-23 clone 1; glycylicycline resistance; comparative genomics

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22 **Objectives:** The whole genomes of two *Acinetobacter baumannii* isolates recovered from a
23 single patient were sequenced to gain insight into the nature and extent of genomic plasticity in
24 this important nosocomial pathogen over the course of a short infection. The first, AB210, was
25 recovered before tigecycline therapy and was susceptible to this agent; the second, AB211, was
26 recovered after therapy and was resistant.

27 **Methods:** DNA from AB210 was sequenced by 454 GS FLX pyrosequencing according to
28 the standard protocol for whole-genome shotgun sequencing, producing ~250-bp fragment reads.
29 AB211 was shotgun-sequenced using the Illumina Genetic Analyzer to produce fragment reads
30 of exactly 36-bp. Single nucleotide polymorphisms (SNPs) and large deletions detected in
31 AB211 in relation to AB210 were confirmed by PCR and DNA sequencing.

32 **Results:** Automated gene-prediction detected 3,850 putative coding sequences (CDS).
33 Sequence analysis demonstrated the presence of plasmids pAB0057 and pACICU2 in both
34 isolates. Eighteen putative SNPs were detected between the pre- and post-therapy isolates,
35 AB210 and AB211. Three contigs in AB210 were not covered by reads in AB211, representing
36 three deletions of approximately 15, 44 and 17 kb.

37 **Conclusions:** This study demonstrates that significant differences were detectable between two
38 bacterial isolates recovered one week apart from the same patient, and reveals the potential of
39 whole-genome sequencing as a tool for elucidating the processes responsible for changes in
40 antibiotic susceptibility profiles.

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43 **Introduction**

44 *Acinetobacter baumannii* is an important nosocomial pathogen, with multidrug-resistant (MDR)
45 and even pan-drug-resistant strains reported world-wide.¹ In the UK, carbapenem-resistant clonal
46 lineages limit available treatment options. One successful lineage, designated OXA-23 clone 1,
47 belonging to European clone II, has been recovered from over 60 hospitals, clustered mainly in
48 London and South-East England.² Representative isolates of this clone are usually susceptible to
49 colistin and tigecycline only. We previously reported the emergence of tigecycline resistance
50 during antibiotic therapy in the OXA-23 clone 1 epidemic lineage, and showed that increased
51 expression of the resistance-nodulation-division (RND) efflux system, AdeABC was responsible
52 for the resistance phenotype.³

53 The recent availability of rapid and inexpensive whole-genome sequencing permits
54 detailed investigation of genetic differences between pairs of bacterial isolates. In *A. baumannii*
55 whole-genome studies have thus far focused either on comparing distinct antibiotic-susceptible
56 and MDR strains,^{4,5} or related isolates from different patients.⁶ The results of these and other
57 similar studies⁷ point to a high degree of genome plasticity, the rapid emergence of antibiotic
58 resistance, and considerable genetic variability even among closely-related isolates.

59 Tigecycline is used as a treatment of last resort for MDR *A. baumannii* infection, despite
60 a lack of formal trial data and the emergence of resistance is a major concern. We sequenced the
61 genomes of two *A. baumannii* isolates from a single patient, the first recovered before tigecycline
62 therapy and susceptible to this agent, the second after one week of therapy for an intra-abdominal
63 infection and resistant. The study aimed to gain insight into the nature and extent of genomic
64 plasticity over the course of a short infection.

65 **Materials and Methods**

66 *Bacterial isolates*

67 Clinical isolates AB210 and AB211 have been described previously.³ As OXA-23 clone 1
68 representatives, they belong to the globally successful European clone II group, and were
69 assigned to Group 1 by the multiplex PCR method described by Turton *et al.*⁸ They were typed
70 by PFGE of *ApaI*-digested genomic DNA (Figure 1), as described previously,² and the presence
71 of *bla*_{OXA-23-like} was confirmed by multiplex PCR.⁹

72 *Antimicrobial susceptibility testing and DNA manipulations*

73 MICs were determined by BSAC agar dilution or Etest (AB bioMérieux, Solna, Sweden) on
74 IsoSensitest agar (Oxiod, Basingstoke, UK) with the results interpreted according to BSAC
75 guidelines.⁹ Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit
76 (Promega, Southampton, UK) and was used as template for DNA sequencing. Plasmids were
77 isolated from AB210 and AB211 using the PureYield Plasmid Miniprep System (Promega) and
78 analysed by agarose gel electrophoresis.

79 *Whole-genome DNA sequencing and data analysis*

80 DNA from AB210 was sequenced by 454 GS FLX pyrosequencing (Roche, Branford,
81 Connecticut, USA) according to the standard protocol for whole-genome shotgun sequencing,
82 producing ~250 bp fragment reads. AB211 was shotgun sequenced using the Illumina Genetic
83 Analyzer (Illumina, Saffron Walden, UK) to produce fragment reads of exactly 36-bp. All
84 sequencing was performed at GATC Biotech Ltd (Constance, Germany). A draft genome
85 assembly for AB210 was produced from flowgram data, using Newbler 2.5 (Roche). The
86 Newbler command-line option ‘-rip’ was used to ensure reads were aligned to single contigs

87 only. The resulting contigs were annotated by reference to the related strain *A. baumannii*
88 ACICU¹⁰ (also belonging to European clone II) using the automated annotation pipeline on the
89 xBASE server.¹¹

90 Illumina reads for isolate AB211 were mapped against the draft AB210 assembly using
91 Bowtie 0.12.0.¹² For the purposes of single nucleotide polymorphism (SNP) detection, Bowtie
92 was run with parameter ‘-m 0’ to suppress alignments that map equally to multiple locations in
93 the genome. To detect deletions this setting was not used. A consensus pileup was produced
94 using SAMtools,¹³ and putative SNPs were called using Varscan 2.2¹⁴ with the following
95 parameters: minimum coverage (10), min-reads2 (2), min-avg-qual (15), min-var-freq (0.9). To
96 detect microindels (insertion or deletion events) less than 3-bases long, AB211 reads were
97 additionally mapped using Novoalign 2.5.¹⁵ Whole-genome alignments were visualised and
98 SNPs and deletions manually inspected using the output files from the above steps using
99 BAMview.¹⁶

100 *Confirmation of SNPs and chromosomal deletions*

101 SNPs and deletions detected in AB211 in relation to AB210 were confirmed by PCR and DNA
102 sequencing using the primers listed in Table S1. Nucleotide sequences of the resulting amplicons
103 were determined with an ABI 3730xl DNA analyser (Applied Biosystems, Warrington, UK).

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108 **Results & Discussion**

109 *Antibiotic susceptibilities*

110 MICs of tigecycline, tobramycin, amikacin, gentamicin and azithromycin for the pre-therapy
111 isolate AB210 were 0.5, >32, >64, >32 and >256 mg/L, respectively, while MICs for the post-
112 therapy isolate AB211 were 16, 2, 4, 8 and >256 mg/L, respectively.

113 *Sequencing results*

114 Sequencing produced >128 million and >156 million sequence reads for AB210 and AB211,
115 respectively. The assembly of AB210 resulted in 91 contigs larger than 500-bp, comprising 4.06
116 megabases of sequence and representing a median 29-fold coverage. Automated gene-prediction
117 detected 3,850 putative coding sequences (CDS), of which 3,504 were homologous (defined as
118 BLASTP e-value $\leq 1e-05$) to a sequence in the reference genome of *A. baumannii* ACICU. The
119 vast majority (96.6 %) of the AB211 reads mapped to a region on the AB210 genome. The
120 AB210 draft assembly has been deposited in GenBank (accession number: AEOX00000000) and
121 raw sequence reads for AB210 and AB211 have been submitted to NCBI's Sequence Read
122 Archive under Study Accession Number SRP004860.

123 *Plasmid profile*

124 Plasmid profiles of AB210 and AB211 were identical and showed the presence of two plasmids
125 in each isolate (data not shown). Sequence analysis demonstrated the presence of a 9-kb contig in
126 AB210 which displayed 99.98 % identity to the previously characterised pAB0057 plasmid.⁵
127 This was seen at high sequence read coverage in both AB210 and AB211, suggesting it was
128 present as multiple copies. Three other contigs, totalling 65 kb, were seen at below-average

129 coverage; taken together these were a full match in length and nucleotide identity to the complete
130 pACICU2 plasmid.¹⁰

131 *AB210 virulence genes and resistance islands*

132 Resistance islands (RIs) have been detected in all sequenced *A. baumannii* genomes containing
133 multiple resistance determinants. They are composite transposons that are complex in nature and
134 which have been designated AbaR (*A. baumannii* resistance).⁴ They share a common insertion
135 site (*comM*) but vary considerably among isolates in terms of the exact genetic composition, with
136 that from ACICU, a representative of European clone II being considerably reduced in size
137 compared to those found in representatives of European clone I.^{10,17} Clinical isolates AB210 and
138 AB211 were found to contain an AbaR-type RI. In the former isolate (GenBank accession
139 number HQ700358) this was shown to contain sequence corresponding to nucleotides 587330-
140 599047 of strain AB0057 (GenBank accession number CP001182), with a 2.85 kb section
141 absent; this is an AbaR4-type island, and contains *bla*_{OXA-23}.

142

143 *SNPs between AB210 and AB211*

144 Eighteen putative SNPs were detected between the pre- and post-therapy isolates. Only one of
145 these was located outside of coding regions at -35 bp upstream of *ureJ* which encodes a
146 hydrogenase/urease accessory protein (AB210 locus tag: AB210-1_2203). The location of this
147 SNP suggests the possibility of regulatory significance although *ureJ* appears to be part of a
148 urease gene cluster which is co-transcribed as an operon in other species.¹⁸ Of the remaining 17,
149 eight were synonymous mutations whereas nine were non-synonymous including one missense
150 mutation (Table 1). Seventeen (94 %) of the SNPs were transitions. Eight of the nine non-

151 synonymous SNPs could be confirmed by PCR and sequencing while one was not validated
152 (Table 1 and Table S1). Several of these were located within genes predicted to be involved in
153 core biological functions, including translation (*dusB*), nucleic acid biosynthesis, α -ketoglutarate
154 and arabinose transport, environmental sensing (the signal transduction histidine kinase gene,
155 *adeS* which had previously been identified through a candidate-gene approach³), and signalling.
156 The mutation in *adeS* is believed to be responsible for up-regulation of the AdeABC efflux
157 system and hence tigecycline resistance. Two SNPs were located within a gene coding for a
158 GGDEF domain-containing protein, one of which was a non-synonymous mutation whilst the
159 other introduced an internal stop codon, thus giving rise to a truncated product (Table 1). These
160 proteins are enzymes that catalyze the synthesis of cyclic-di-GMP, which has been recognized
161 recently as an important second messenger in bacteria and is implicated in adhesin and
162 extrapolsaccharide biosynthesis.¹⁹

163 *Large structural changes in the genomes of AB210 and AB211*

164 Three contigs in AB210 were not covered by reads in AB211, these putative deletions were
165 designated ROD1, 2 and 3. The first, ROD1, was approximately 15 kb in length. This deletion
166 disrupted the coding sequence of the DNA mismatch repair gene *mutS* (AB210-1_2445) by
167 eliminating the N-terminal *mutS-I* domain. Aside from encoding this mismatch recognition
168 enzyme, ROD1 also encoded a DMT superfamily permease (AB210-1_2447) and an MFS
169 permease (AB210-1_2451), transcriptional regulators (AB210-1_2450; AB210-1_2453), an EAL
170 domain-containing protein (AB210-1_2448), responsible for the degradation of cyclic-di-GMP.¹⁹
171 At approximately 44 kb ROD2 was the largest deleted region and comprised of genes encoding
172 for transcriptional regulators (AB210-1_3253; AB210-1_3262; AB210-1_3269; AB210-
173 1_3273), ion channels and transporters (AB210-1_3254; AB210-1_3259; [AB210-1_3275;

174 AB210-1_3276; AB210-1_3277]), a class A β -lactamase enzyme (AB210-1_3248) and
175 components of a type VI secretion system (AB210-1_3280; AB210-1_3281).²⁰ Interestingly, part
176 of the type VI secretion locus was missing even in AB210, suggesting that this was a degenerate
177 system in both isolates. ROD1 and ROD2 are contiguous in *A. baumannii* ACICU, suggesting
178 this may be a single deletion, but this could not be confirmed experimentally for AB210 by PCR
179 (data not shown). ROD3, approximately 17 kb in length, included a class 1 integron containing
180 antibiotic resistance genes including macrolide resistance determinants (AB210-1_3691
181 [phosphotransferase]; AB210-1_3692 [an efflux protein]) and several genes encoding
182 aminoglycoside resistance determinants, namely *aac(6')-Ib* (AB210-1_3701), two copies of
183 *aadA* (AB210-1_3699; AB210-1_3700) and *armA* (AB210-1_3695), which encodes a 16S rRNA
184 methylase.

185

186 *Implications for Acinetobacter evolution*

187 The extent of genomic changes detected here are consistent with the marked changes in
188 phenotype, particularly the loss of aminoglycoside resistance in AB211. However, we were
189 unable to determine whether these changes were the result of rapid evolution during the course
190 of infection and treatment, or whether the patient initially had a mixed infection (or re-infection),
191 involving different variants of the same defined clone, with subsequent selection for tigecycline
192 resistance.

193 The disruption of *mutS*, an important DNA mismatch repair gene, is significant and
194 suggests the possibility of a hypermutator phenotype, which may have contributed to the
195 relatively large number of SNPs. Previous work in *Acinetobacter* sp. ADP1 has shown that *mutS*

196 preferentially recognises and repairs transitions,²¹ so its disruption in AB211 is consistent with
197 our observation that 94 % of the SNPs belonged to this class.

198 The absence of ROD3 is consistent with the change in aminoglycoside resistance
199 between AB210 and AB211, with MICs of tobramycin, amikacin and gentamicin reduced at
200 least 8-fold in AB211. It is notable that the development of tigecycline resistance was
201 accompanied by increased susceptibility to other antibiotics through a large genomic deletion.

202 GGDEF and EAL-containing proteins have been implicated in sessile to planktonic
203 shifts. Taken together, the termination in a GGDEF domain-containing protein as well as the loss
204 of an EAL-domain containing protein in ROD1 may be advantageous during the process of
205 infection though this remains to experimentally determined.

206 In this study, whole-genome sequencing gave insight into the nature of genetic changes
207 between isolates under selection pressure through antibiotic therapy and a hostile host
208 environment. This study has demonstrated significant differences between two *A. baumannii*
209 isolates belonging to the same epidemic lineage, collected one week apart from the same patient.
210 Such studies are able to shed light on the relative importance of SNPs and transposon
211 mutagenesis on the evolution of *A. baumannii* and can generate hypotheses into the nature of
212 antibiotic resistance and virulence. Although further studies are needed to assess the extent of
213 genetic diversity among populations of *A. baumannii* in a single patient, we clearly demonstrated
214 the potential of whole-genome sequencing as an important tool for helping elucidate the
215 evolutionary processes responsible for the rapid development of antibiotic resistance in this
216 important nosocomial pathogen.

217

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220 analysis.

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223

224 **Transparency Declarations**

225 D. M. L. has (i) received research grants from Wyeth and Pfizer, (ii) spoken at meetings
226 organised by Wyeth and Pfizer, (iii) received sponsorship to travel to congresses from Wyeth
227 and Pfizer, as well as from numerous other pharmaceutical and diagnostic companies. He holds
228 shares in GlaxoSmithKline, Merck, AstraZeneca, Dechra and Pfizer; he acts also as Enduring
229 Attorney for a close relative, managing further holdings in GlaxoSmithKline and EcoAnimal
230 Health. N. W. has received research grants from Wyeth. M. E., M. D., J. F. T., A. U., T. G., D.
231 M. L. and N. W. are employees of the HPA and are influenced by its views on antibiotic use and
232 prescribing. M. H., D. W. W. and C. P. T. have received sponsorship to attend conferences from
233 Wyeth. N. L. and M. J. P. : none to declare.

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- 289
- 290

Table 1. Confirmed SNPs identified in clinical isolate AB211 resulting in amino acid substitution or termination

SNP	Position in AB210 assembly	Locus tag in AB210 assembly	Protein product	Amino acid identity	
				AB210	AB211
1	159509	AB210-1_0138	tRNA-dihydrouridine synthase, DusB	A	T
2	639321	AB210-1_0587	nucleoside-diphosphate-sugar epimerase	T	A
3	755474	AB210-1_0703	major facilitator superfamily permease	V	A
4	1469178	AB210-1_1405	hypothetical protein	A	V
5	2548057	AB210-1_2423	major facilitator superfamily permease	A	T
6	2852737	AB210-1_2721	Signal transduction histidine kinase, AdeS	A	V
7	3362158	AB210-1_3207	GGDEF domain-containing protein	Q	*
8	3362175	AB210-1_3207	GGDEF domain-containing protein	G	V

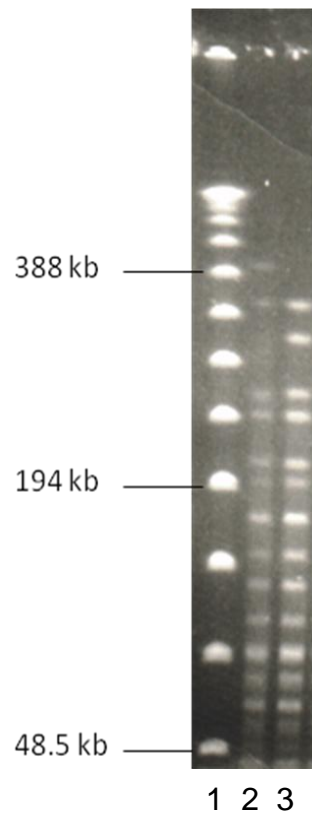


Figure 1.

Figure Legends

Figure 1. PFGE profiles of AB210 (lane 2) and AB211 (lane 3).