
RCH51, a multiply antibiotic-resistant *Acinetobacter baumannii* ST103IP isolate, carries resistance genes in three plasmids, including a novel potentially conjugative plasmid carrying *oxa235* in transposon Tn6252

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Objectives: To determine the identity and context of genes conferring antibiotic resistance in a sporadic multiply antibiotic-resistant *Acinetobacter baumannii* recovered at Royal Children's Hospital, Brisbane.

Methods: The antibiotic resistance phenotype for 23 antibiotics was determined using disc diffusion or MIC determination. The whole-genome sequence of RCH51 was determined using the Illumina HiSeq platform. Antibiotic resistance determinants were identified using ResFinder. Plasmids were recovered by transformation.

Results: Isolate RCH51 belongs to the uncommon STs ST103_{IP} (7-3-2-1-7-1-4) and ST514_{OX} (1-52-29-28-18-114-7). It was found to be resistant to sulfamethoxazole, tetracycline, gentamicin, tobramycin and kanamycin and also exhibited reduced susceptibility to imipenem (MIC 2 mg/L) and meropenem (MIC 6 mg/L). RCH51 carries the *oxa235*, *sul2*, *floR*, *aadB* and *tet39* resistance genes, all located on plasmids. The largest of the three plasmids, pRCH51-3, is 52 789 bp and carries *oxa235* in the ISAb1-bounded transposon Tn6252, as well as *sul2* and *floR*. pRCH51-3 represents a new *A. baumannii* plasmid family that is potentially conjugative as it contains several genes predicted to encode transfer functions. However, conjugation of pRCH51-3 was not detected. The *aadB* and *tet39* resistance genes were each found in small plasmids identical to the known plasmids pRAY*-v1 and pRCH52-1, respectively.

Conclusions: The resistance gene complement of RCH51 was found in three plasmids. pRCH51-3, which carries the *oxa235*, *sul2* and *floR* resistance genes, represents a new, potentially conjugative *A. baumannii* plasmid type.

Introduction

Carbapenem antibiotics have been the frontline treatment option for infections caused by multiply antibiotic-resistant *Acinetobacter baumannii*. However, resistance to carbapenems in *A. baumannii* is now widespread globally.¹ In *A. baumannii*, carbapenem resistance is most often caused by class D β -lactamases (oxacillinases), while class B enzymes (metallo- β -lactamases) are still rare.² To date, five types of oxacillinase have been associated with *A. baumannii*, OXA-23, OXA-24 (OXA-40) and OXA-58² as well as more recently identified and rare enzymes, OXA-143³ and OXA-235.⁴ The intrinsic *A. baumannii* *oxaAb* gene, encoding an OXA-51-like enzyme, does not significantly contribute to carbapenem resistance unless it is activated by an IS.⁵

The 831 bp *oxa235* gene, first described in 2013, causes reduced carbapenem susceptibility.⁴ It was found in eight *A. baumannii* isolates recovered in the USA and Mexico between 2007 and 2009.⁴ The original sequence of *oxa235* was flanked by

two partial copies of ISAb1, suggesting it is part of a transposon,⁴ and this 3267 bp composite transposon, designated Tn6252, was later found in the chromosome of *A. baumannii* LAC-4 (GenBank accession number CP007712).⁶ Tn6252 consists of a 907 bp central DNA segment (including *oxa235*) bounded by inversely oriented copies of ISAb1 and flanked by a 9 bp target site duplication (TSD).⁶

Here, we have characterized the context of the resistance genes responsible for the antibiotic resistance phenotype of an Australian multiply antibiotic-resistant *A. baumannii* strain that carries *oxa235*.

Materials and methods

Antibiotic resistance testing

The antibiotic resistance profile of RCH51 was determined using a disc diffusion method as described previously.⁷ MICs of imipenem and meropenem

were determined using Etest strips (bioMérieux, Pty Ltd) according to the manufacturer's instructions.

WGS, plasmid assembly and annotation

Genomic DNA isolated from RCH51 was sequenced using Illumina HiSeq. Paired-end reads of 100 bp were assembled using Velvet (v. 1.2.10) yielding 80 contigs with an average depth of 220-fold. The sequence types (STs) in the Institut Pasteur and Oxford MLST schemes (http://pubmlst.org/abau_mannii/) were determined from the genome sequence data as described previously.⁸

Antibiotic resistance genes and contigs carrying them were identified using ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>), and recovered using standalone BLAST (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast/LATEST/>). PCR primers were designed facing outwards from each contig and sequencing of the resulting amplicons joined the contigs. pRCH51-3 was assembled from three contigs of 44 623, 1088 and 4964 bp with fragments of ISAb_{a1} on both ends. Plasmid copy numbers were estimated by dividing the coverage of plasmid contigs by that of chromosomal contigs.

Plasmid sequences were annotated using Prokka⁹ followed by manual modification, using Pfam (<http://pfam.xfam.org/>), ISFinder (<https://www-is.biotoul.fr/>) and BLASTp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) searches to identify additional features. GC skew and GC plots were drawn using DNA Plotter (v. 10.2) (<http://www.sanger.ac.uk/science/tools/dnaplotter>). Figures were drawn to scale using Gene Construction Kit (GCK 4.0.3), SnapGene[®] Viewer 2.8.1 and Adobe Illustrator CS6.

Transformation and conjugation

Plasmids were transformed into *A. baumannii* AB307-0294¹⁰ made electrocompetent as previously described¹¹ using cells grown at 37°C to exponential phase (OD₆₀₀ 0.5–0.7). Electrocompetent AB307-0294 (801 L) was mixed with 150 ng or 1.5 µg of plasmid DNA in 0.2 cm cuvettes and electroporation was performed using the following parameters: 2.5 kV, 25 IF, 200 X. L-agar containing kanamycin (20 mg/L) or tetracycline (4 mg/L) and Mueller–Hinton agar (MHA) containing sulfamethoxazole (100 mg/L) were used to select for transformants.

For conjugation experiments, equal amounts of overnight cultures of the donor RCH51 and the nalidixic acid resistant recipient AB307-0294 were mixed and incubated on an L-agar plate overnight. MHA plates containing nalidixic acid (25 mg/L) and sulfamethoxazole (100 mg/L) were used to select for transconjugants.

Nucleotide accession number

The complete sequence of pRCH51-3 has been deposited in the GenBank database under accession number KY216144.

Results and discussion

RCH51, a sporadic multiply antibiotic-resistant *A. baumannii* strain recovered at the Royal Children's Hospital in Brisbane prior to 2010, belongs to ST514_{OX} (1-52-29-28-18-114-7) and ST103_{IP} (7-3-2-1-7-1-4). These are rare STs as only three strains, from the USA, Egypt and Brazil, belonging to either of them were found in the *A. baumannii* MLST databases. RCH51 was found to be resistant to sulfamethoxazole, tetracycline, gentamicin, tobramycin and kanamycin and exhibited reduced susceptibility to imipenem (MIC 2 mg/L) and meropenem (MIC 6 mg/L). RCH51 was susceptible to ceftazidime, cefotaxime, nalidixic acid and ciprofloxacin.

Using ResFinder, the *aadB*, *tetA39*, *sul2* and *oxa235* genes were found in the draft genome of RCH51, accounting for resistance to

gentamicin, kanamycin and tobramycin (*aadB*), tetracycline (*tetA39*), sulphonamides (*sul2*) and *oxa235* accounts for the reduced susceptibility to imipenem and meropenem, respectively. The chloramphenicol and florfenicol resistance gene *floR* was also found in RCH51 but *A. baumannii* strains exhibit high-level chloramphenicol resistance due to the intrinsic *craA* gene encoding an efflux pump.¹²

The resistance genes were localized to three plasmids (Table 1), each of which could be recovered by transformation. The *oxa235* gene together with the *sul2* and *floR* genes were localized to the largest plasmid, pRCH51-3. The contigs containing the resistance genes had a higher coverage, compared with those containing standard chromosomal genes (Table 1).

Tn6252 carrying *oxa235*

The *oxa235* gene was found in the ISAb_{a1}-bounded transposon Tn6252, flanked by the 9 bp TSD 'TTTTCTTTA'. In RCH51, Tn6252 is located in a 52 789 bp plasmid, pRCH51-3. In LAC-4 the TSD flanking Tn6252 is 'ATTATTTA', and Tn6252 was also found in a third location in a 15 kb *A. baumannii* plasmid pORAB01-3 (GenBank accession number CP015486) flanked by 'GTAGATTTT'. The three locations confirm that the *oxa235* gene is moved by Tn6252. Tn6252 resembles Tn2006¹³ in that both include inversely oriented ISAb_{a1} copies.

In Tn6252, ISAb_{a1} is located 14 bp upstream of the *oxa235* gene in the orientation that provides a strong promoter to enhance its expression.¹⁴ pRCH51-3 was electroporated into *A. baumannii* AB307-0294 (imipenem MIC 0.25 mg/L; meropenem MIC 0.38 mg/L), and the two transformants tested exhibited only reduced susceptibility to imipenem (MIC 2–3 mg/L) and meropenem (MIC 5–6 mg/L). Given the strong promoter and that the copy number of pRCH51-3, relative to the chromosome, was 3–4 (Table 1), it is likely that a chromosomally located *oxa235* gene with no upstream ISAb_{a1} would not contribute significantly to carbapenem resistance.

pRCH51-3 carrying *oxa235* in Tn6252, *sul2* and *floR*

The *sul2* sulphonamide resistance gene is located between a fragment of ISAb_{a1} (300 bp) and a fragment of CR2 (1050 bp), which is present 346 bp downstream of *sul2* (Figure 1). Although this segment resembles the region surrounding *sul2* in the ISAb_{a1}-interrupted variant of the GIsul2 genomic island,¹⁵ in pRCH51-3 ISAb_{a1} is 18 bp upstream of *sul2*, while this distance is 21 bp in ATCC 17978. Hence, ISAb_{a1} may have inserted upstream of *sul2* more than once. The *floR* gene is also located between part of a novel IS (ISAb_{a2}-like) and an ORF encoding a LysR family transcription regulator. The FloR protein shares 99% amino acid identity with the well-characterized florfenicol/chloramphenicol efflux pump (399/404 aa) from *Salmonella* genomic island 1 (SGI1; GenBank accession number AF261825). However, the *floR* gene in pRCH51-3 is in a completely different context.

The closest match in the GenBank non-redundant database is part of an *A. baumannii* plasmid, pAB (GenBank accession number HQ917128), which is identical to the 2667 bp segment between the partial ISAb_{a2}-like and ISAb_{a1} sequences in pRCH51-3. This segment is also part of a larger region found in the plasmids of several different species, e.g. the *Escherichia coli* plasmid pYD786-2 (GenBank accession number KU254579).

Table 1. Properties of plasmids found in RCH51

Plasmid	Equivalent to ^a	Resistance gene	Length (bp)	Copy number ^c
pRCH51-1	pRAY*-v1	<i>aadB</i>	6078	15–16
pRCH51-2	pRCH52-1	<i>tet39</i>	11164	6
pRCH51-3 ^b	—	<i>oxa235</i> (in Tn6252), <i>sul2</i> , <i>floR</i>	44623	3–4

^apRAY*-v1 (GenBank accession number JF343536), pRCH52-1 (GenBank accession number KT346360).

^bGenBank accession number KY216144.

^cEstimated by dividing the average coverage of plasmid contigs by that of chromosomal contigs.

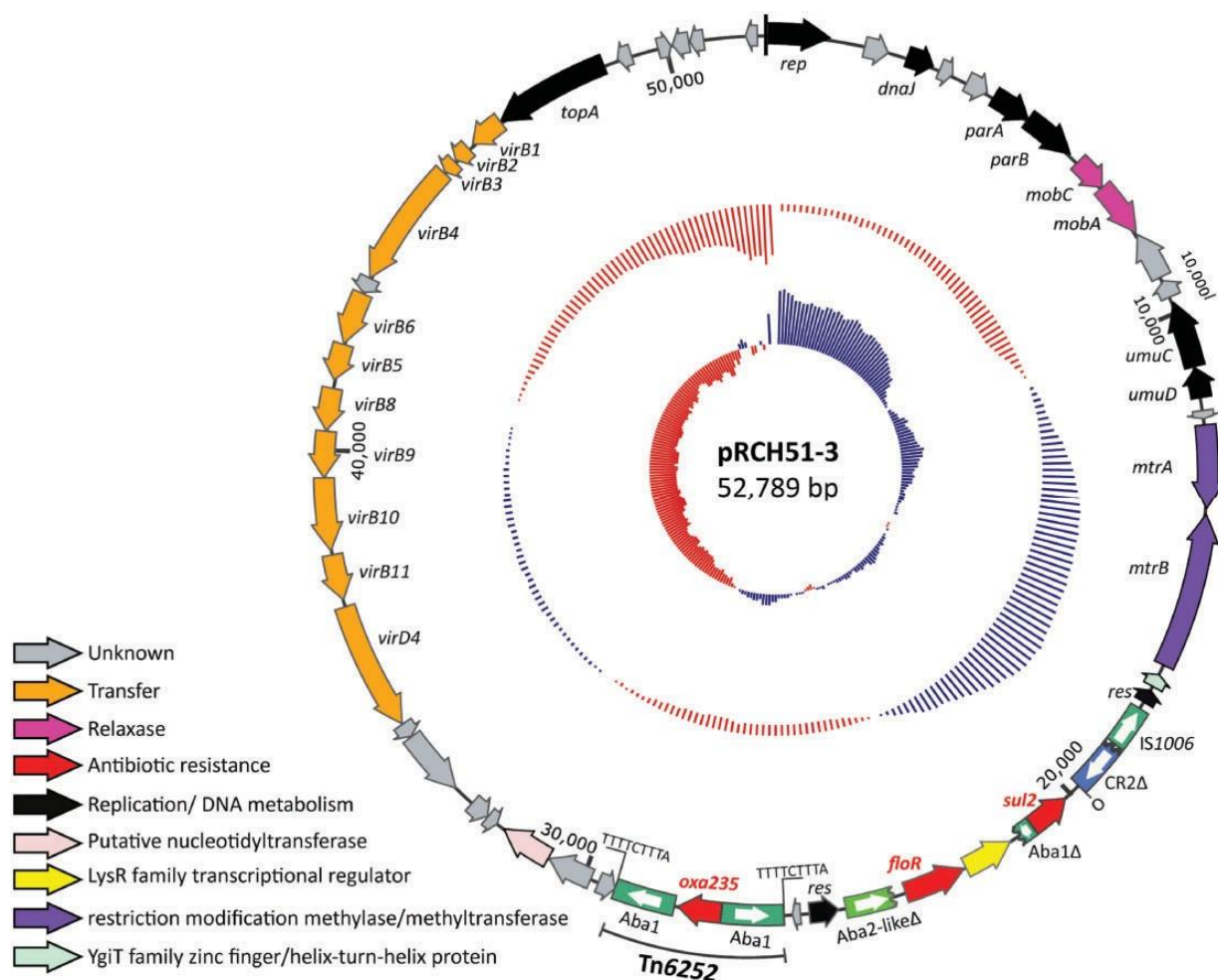


Figure 1. Circular map of pRCH51-3. The outermost ring represents the pRCH51-3 plasmid with arrows indicating genes/ORFs involved in the various cellular pathways. Functions of the genes and ORFs found are colour coded and shown in the left bottom corner of the figure. ISs are shown using green boxes and the orientation of the *tnp* genes is indicated using white arrows inside the boxes. The middle ring and the innermost ring represent a GC plot and a GC skew plot, respectively. For both plots, red and dark blue indicate the measures below and above the average, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

***pRCH51-3* represents a new *Acinetobacter* plasmid family**

pRCH51-3 encodes a novel Rep protein belonging to the Rep-3 replication initiation protein family (Pfam01051). This Rep shares 98% identity with that of an *Acinetobacter nosocomialis*

plasmid (p6411-66.409kb; GenBank accession number CP010903), but the closest known Rep is RepAci5, which only shares 27% amino acid identity across 61% of the protein length. This suggests that these Rep proteins represent a novel plasmid family. pRCH51-3 also encodes potential partitioning

proteins ParA and ParB, a TopA topoisomerase and a UmuCD translesion polymerase, which is involved in DNA repair after DNA damage (Figure 1).¹⁶ In addition, a set of genes predicted to encode conjugative transfer functions was found in pRCH51-3, suggesting that this plasmid may be transferable. These regions share 98% DNA identity with the corresponding parts of the *A. nosocomialis* plasmid p6411-66.409kb. The products of transfer genes found in pRCH51-3 are distantly related (with an average of 30% amino acid identity) to the conjugative transfer proteins of the pTi plasmid, the exemplar of the MPF_T transfer system.¹⁷ However, VirB7 (lipoprotein) was not found in pRCH51-3 (Figure 1). This suggested that pRCH51-3 may be conjugative but several attempts to transfer it by conjugation into AB307-0294 failed.

No other plasmids matching the pRCH51-3 backbone and p6411-66.409kb were detected in the GenBank non-redundant or WGS databases. Hence, pRCH51-3 represents a new plasmid type (Figure 1).

Small plasmids carrying *aadB* and *tetA39* genes

The *aadB* gene was found in a 6078 bp plasmid named pRCH51-1 (copy number 15–16; Table 1). pRCH51-1 differs by 1 bp, in a non-coding region, from pRAY*-v1 (GenBank accession number JF343536).¹⁸ Plasmid pRAY* is widespread in *Acinetobacter* spp., but the pRAY*-v1 variant, which differs from pRAY* (GenBank accession number JQ904627) by 65 single base changes clustered within a 1358 bp region of the putative mobilization genes,¹⁸ has been seen previously only in Australian global clone 2 (GC2) strains.⁷ Hence, this is the first time that pRAY*-v1 has been seen outside the GC2 clone.

The *tetA39* and adjacent *tetR39* genes were in an 11164 bp plasmid, pRCH51-2 (copy number 6; Table 1), which is identical to the recently described pRCH52-1.¹¹ As both RCH51 and RCH52 (carrying pRCH52-1) were recovered at the same hospital, this plasmid may have spread in that institution.

Conclusions

The sporadic strain RCH51 has become multiply resistant by acquiring three plasmids, two known and one novel, carrying resistance genes.

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Transparency declarations

None to declare.

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