

# Cl415, a carbapenem-resistant *Acinetobacter baumannii* isolate containing four AbaR4 and a new variant of AbGRI2, represents a novel global clone 2 strain

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**Objectives:** To determine the genetic context of genes conferring antibiotic resistance on the carbapenem-resistant *Acinetobacter baumannii* Cl415, recovered in 2017 at El Youssef Hospital Centre in Akkar Governorate, North Lebanon.

**Methods:** Antibiotic resistance phenotype for 22 antibiotics was determined using disc diffusion or MIC determination. The whole-genome sequence of Cl415 was determined using a combination of the Illumina MiSeq and Oxford Nanopore (MinION) platforms. Complete genome was assembled using Unicycler and antibiotic resistance determinants and ISs were identified using ResFinder and ISFinder, respectively.

**Results:** Cl415 is a global clone 2 (GC2) strain and belongs to the most common STs of this clone, ST<sub>21P</sub> and ST<sub>218OX</sub>. Cl415 is resistant to several antibiotics, including aminoglycosides and carbapenems to a high level. Genomic analysis of Cl415 revealed that it carries four chromosomal AbaR4 copies. One copy was found in the *comM* gene replacing the AbGRI1 island. Cl415 also contains a novel variant of AbGRI2, herein called AbGRI2-15, carrying only the *bla*<sub>TEM</sub> and *aphA1* resistance genes. Cl415 belongs to a subclade of GC2 strains that appear to have diverged recently with a wide geographical distribution.

**Conclusions:** The resistance gene complement of Cl415 was found in the chromosome with four *oxa23* located in AbaR4 copies and the remaining genes in a novel variant of the AbGRI2 resistance island. Cl415 was isolated in Lebanon, but phylogenetic analysis suggests that Cl415 represents a new lineage with global distribution within GC2.

## Introduction

Treatment of infections due to carbapenem-resistant *Acinetobacter baumannii* (CRAb) has become a major concern due to the global spread of XDR strains that are also resistant to carbapenems.<sup>1,2</sup> In *A. baumannii*, carbapenem resistance is most often caused by class D  $\beta$ -lactamases (oxacillinases) with OXA-23, OXA-24 (OXA-40) and OXA-58 considered the most prevalent enzymes, while class B enzymes (MBLs) are still rare.<sup>2,3</sup>

The majority of CRAb strains belong to two major global clones, namely global clone 1 (GC1) and global clone 2 (GC2) (also known as international clones; ICs),<sup>4</sup> with GC2 being by far the most widespread clone in most countries.<sup>2,5</sup> GC1 strains often carry variants

of a large genomic island (GI) called an AbaR, which consists of a backbone transposon Tn6019 and a variable central multiple antibiotic resistance region (MARR) located between two copies of Tn6018 and in the chromosomal *comM* gene.<sup>6</sup> However, we previously showed that a subclade of GC1 strains in lineage 2 have diverged by replacing AbaRs with AbaR4, which is a different but related GI that carries the *oxa23* carbapenem resistance gene in an ISAba1-bounded transposon called Tn2006.<sup>7,8</sup>

In GC2 strains, the AbGRI1 island and its variants are found in *comM*.<sup>9,10</sup> AbGRI1 has substantial structural differences when compared with AbaRs in GC1 strains.<sup>11</sup> The AbGRI1 ancestral version (AbGRI1-0) was predicted to consist of a copy of Tn6022

(the backbone of AbaR4) at the left-hand end, a central segment derived from the backbone of a plasmid like pAB3 (found in ATCC 17978 GenBank accession number CP012005), and a copy of Tn6172 at the right-hand end.<sup>9,10,12</sup> Tn6172 contains a transposition module related to those in Tn6022 (AbaR4 backbone), Tn6019 (backbone of AbaR-type resistance islands) and a segment containing the *sul2* sulphonamide and *strAB* streptomycin/spectinomycin resistance genes.<sup>9,10,12</sup> GC2 strains also often have a second GI called AbGRI2, which is an IS26-bounded resistance island.<sup>11</sup> This GI often carries several resistance genes including *aphA1* (kanamycin and neomycin resistance gene), *catA1* (chloramphenicol resistance gene) and a class 1 integron containing *sul1*, *aadA1* and *aacC1* that confer resistance to sulphonamides, streptomycin/spectinomycin and gentamicin, respectively.<sup>11</sup> A subset of GC2 strains contains a third antibiotic resistance island called AbGRI3, which is another IS26-bounded GI containing the *armA* gene that confers resistance to all clinically relevant aminoglycosides.<sup>11,13</sup> An additional island called AbGRI4, which is a class 1 integron bounded by IS26, has also been reported in GC2 strains and non-GC2 strains.<sup>14</sup>

Here, we have characterized the genetic context of the resistance genes responsible for the antibiotic resistance phenotype of a Lebanese XDR *A. baumannii* strain and showed it carries four copies of AbaR4 and a novel variant of the AbGRI2 island in its chromosome. Cl415 has recently diverged and represents a new GC2 strain.

## Material and methods

### Bacterial strain and antibiotic resistance testing

*A. baumannii* Cl415 was recovered 24 days after hospital admission from the blood sample of a 71-year-old female inpatient suffering from stroke-like symptoms at the ICU of El Youssef Hospital Centre in Akkar Governorate, North Lebanon in July 2017.

The antibiotic resistance profile and the MIC of meropenem and colistin were determined using the disc diffusion and standard microbroth dilution methods as described previously.<sup>15,16</sup> Resistance profiles were interpreted according to the CLSI guidelines for *Acinetobacter* spp.<sup>17</sup> and the calibrated dichotomous sensitivity (CDS) disc diffusion assay (<http://cdstest.net>) when a CLSI breakpoint was not available (e.g. for netilmicin, streptomycin, spectinomycin, sulfamethoxazole, nalidixic acid and rifampicin). *A. baumannii* D36, containing a single copy of *oxa23*,<sup>7</sup> was used as internal control in MIC assays.

### WGS, genome assembly, annotation and phylogenetic analysis

Genomic DNA isolated from a single colony of Cl415 using the DNeasy™ UltraClean Microbial Kit (QIAGEN, Germantown, MD, USA) was sequenced using Illumina MiSeq and Oxford Nanopore (MinION), as described previously.<sup>18,19</sup> Illumina sequencing generated 4 447 132 paired-end short reads with 141-fold coverage and an average length of 250 bp. MinION generated a total of 47 789 reads and 60-fold coverage. High-quality Illumina and MinION reads were assembled *de novo* using a hybrid assembly approach using Unicycler (v0.4.7).<sup>20</sup>

Protein coding, rRNA and tRNA gene sequences were annotated automatically using Prokka (v.1.13),<sup>21</sup> followed by detailed manual annotations of resistance regions, surface polysaccharide loci and ISs, using ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>), ISFinder (<https://www-is.bio.toul.fr/>), Kaptive (v.0.2) (<https://kaptive-web.erc.monash.edu/>) and Pfam (<http://pfam.xfam.org/>) searches. MLST (Institut Pasteur and Oxford

schemes) STs (<http://pubmlst.org/abumannii/>) were determined from the genome sequence data as described previously.<sup>22</sup> Figures were drawn to scale using SnapGene® (v.5.2.4) and Inkscape (v.1.0).

To examine the relationship of Cl415 to other GC2s, all complete GC2 genomes (139 entries, as of April 2021) were downloaded and used to construct a recombination-free whole-genome phylogenetic tree, as previously described in detail.<sup>23</sup> The phylogenetic tree was visualized and plotted using the ggtree package in R (v.4.0.5).

### Quantitative real-time PCR (qRT-PCR), data normalization and expression-level changes

The relative expression level of *oxa23* was measured using a qRT-PCR assay using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA) as previously described.<sup>24</sup> The cDNAs were quantified on a QuantStudio Flex 6 Real-Time PCR System (Applied Biosystems) using the cycling conditions described before.<sup>24</sup> The 16S rRNA primers were those previously designed<sup>24</sup> and MH39: 5'-ACCTGCTGTCCAATTCAGC-3' and MH40: 5'-AAGGGCGAGAAAAGGTCATT-3' (generating a 173 bp segment of *oxa23*) were designed in this study. The 16S rRNA gene was used as an internal control as well as strain D36 (GenBank accession number CP012952) with a single *oxa23* copy (in AbaR4; see results) to compare expression levels.

### Nucleotide accession numbers

The complete genome sequence of Cl415 has been deposited in the GenBank/EMBL/DDBJ database under accession numbers CP071763 (chromosome), CP071764 (p1Cl415) and CP071765 (p2Cl415). Illumina and MinION reads are deposited under SRR14534402 and SRR14534401, respectively.

## Results and discussion

### Antibiotic resistance phenotype

Cl415 is resistant to carbapenems (imipenem and meropenem), third-generation cephalosporins (cefotaxime, ceftazidime and ceftriaxone), kanamycin, neomycin, gentamicin, streptomycin, spectinomycin, chloramphenicol, nalidixic acid and ciprofloxacin, making it an XDR strain (Table S1, available as [Supplementary data](#) at JAC Online). Cl415 is susceptible to colistin (MIC ≤ 0.5 mg/L).

### Complete genome of Cl415, antibiotic resistance genes, ST and surface polysaccharides

The genome of Cl415 was completed using a combination of Illumina MiSeq and Oxford Nanopore (MinION) data using a hybrid assembly approach. It consists of a 3 965 259 bp chromosome and two cryptic plasmids, p1Cl415 (2277 bp) and p2Cl415 (8731 bp). p1Cl415 contains a single reading frame encoding a putative replication initiation protein that is 59.55% aa identical to RepAci5 (GenBank accession number GU978999). p2Cl415 encodes RepAci1 and is identical to pA1-1 (GenBank accession number CP010782), which is widely spread in GC1 and GC2 strains.

The Cl415 genome contains four copies of the *oxa23* carbapenem resistance gene, *bla*<sub>TEM</sub> (β-lactam resistance) and *aphA1* (kanamycin and neomycin resistance), accounting for the observed resistance phenotype. A copy of ISAba1 was found, upstream of the chromosomal *ampC* gene, accounting for the Cl415 resistance to third-generation cephalosporins, as previously described.<sup>25,26</sup> Cl415 is resistant to fluoroquinolones due to the mutations found in the *gyrA* DNA gyrase and *parC* topoisomerase

IV genes, leading to GyrA (S81L) and ParC (S84L) substitutions. These specific mutations are well known to cause resistance to fluoroquinolones in Gram-negative bacteria including *A. baumannii* (e.g. nalidixic acid).<sup>27,28</sup>

Cl415 encodes the OXA-66 variant of the intrinsic *oxaAb* gene. It belongs to STs ST218<sub>OX</sub> (*gltA-1*, *gyrB-3*, *gdhB-3*, *recA-2*, *cpn60-2*, *gpi-102*, *rpoD-3*) and ST2<sub>IP</sub> (*cpn60-2*, *fusA-2*, *gltA-2*, *pyrG-2*, *recA-2*, *rplB-2*, *rpoB-2*). Hence, Cl415 is a GC2 strain. The bacterium encodes the KL9 and OCL1 surface polysaccharides, which have been previously reported in GC2 isolates, e.g. in MDR-TJ (GenBank accession number NC\_017847).

#### Four *oxa23* copies associated with high carbapenemase expression and high MICs

Here, we used qRT-PCR and MIC testing to examine whether the detected multiple copies of *oxa23* in Cl415 are associated with high expression levels of the gene and high carbapenem MICs. The meropenem MIC was found to be 128 mg/L (versus 16 mg/L for the D36 strain with a single *oxa23* copy). The meropenem MIC correlated well with a 3.74-fold increase in the *oxa23* expression level compared with the control (D36 with one AbaR4 copy). Differences in Cl415 and D36 MICs and *oxa23* expression levels could be due to different genetic backgrounds but the *oxa23* gene is in the same transposon in both strains (AbaR4 is not commonly present in the chromosome of GC2 strains, therefore D36 was used as a closest available strain with a chromosomal AbaR4). However, regardless of the genetic background and the strain used for comparison (D36), the meropenem MIC of 128 mg/L observed in Cl415 is significant considering the CLSI-recommended threshold of  $\geq 8$  mg/L for meropenem resistance. Here, the observed elevated MIC and transcript level indicates the cumulative *oxa23* expression involving all four copies. It is known that the expression of the *oxa23* gene is primarily driven by the ISAbA1 copy present upstream of this gene and given that all four *oxa23* copies are in Tn2006 and all Tn2006 copies are in AbaR4, it is likely that all copies have approximately similar expression level. However, determining the exact role of each copy requires further examination to generate single-, double- and triple-*oxa23* mutants of Cl415. This was not further pursued here.

Amplification of several resistance genes has also been shown to contribute to increased expression and therefore resistance phenotypes.<sup>29,30</sup> A previous study showed that an increased *oxa23* copy number could lead to higher transcript levels and MIC of carbapenem in *A. baumannii*<sup>29</sup> while a 2016 study did not find any correlation between *oxa23* amplification and enhanced carbapenem resistance.<sup>31</sup> Here, consistent with Zhang *et al.*,<sup>29</sup> we show that the increased *oxa23* copy numbers could lead to increased *oxa23* expression level and therefore higher meropenem MICs.

#### Cl415 carries four chromosomal AbaR4 islands, one in *comM* replacing the AbGRI1 island

Analysis of the Cl415 genome showed that it contains four *oxa23* copies, with each copy in Tn2006 located in an AbaR4 resistance island (bases 1 049 485–1 066 296, 1 232 777–1 249 588, 2 188 320–2 205 131 and 3 706 090–3 722 901 of CP071763). One of the AbaR4 copies is in the *comM* gene, precisely where an AbGRI1 resistance island would be present (Figure 1a). All of the other three AbaR4 insertions are in unique chromosomal locations

as these regions are intact in other publicly available complete GC2 strains. It is likely that AbaR4 was initially inserted in *comM* in Cl415, or its recent progenitor, then moved to three additional chromosomal locations.

We previously reported that AbaR4 replaces the AbaR-type island in a GC1 strain (isolate D36 imported to Australia via military personnel),<sup>7</sup> which was later found to be related to a set of Middle Eastern strains that all carry an AbaR4 in *comM*.<sup>23</sup> Here, we searched the GenBank non-redundant database (April 2021) and found several unrelated *Acinetobacter* strains that contain an AbaR4 in *comM* (Table 1), indicating that AbaR4 can target *comM* in non-*baumannii* strains. Amongst this set, there is a GC2 strain, recovered in 2015 (strain ABUH773; GenBank accession number CP035049), that was recently reported to contain AbaR4 in *comM*,<sup>14</sup> suggesting a possible relationship with Cl415. However, phylogenetic analysis did not confirm this (see below). Recently, AbaR4 was also reported in *comM* in three *Proteus mirabilis* strains, indicating that the transposon backbone of AbaR4, the Tn6022, can target different bacterial genera, thus highlighting its significance as an active transposon that can spread the *oxa23* carbapenem resistance gene beyond *Acinetobacter*.<sup>32</sup>

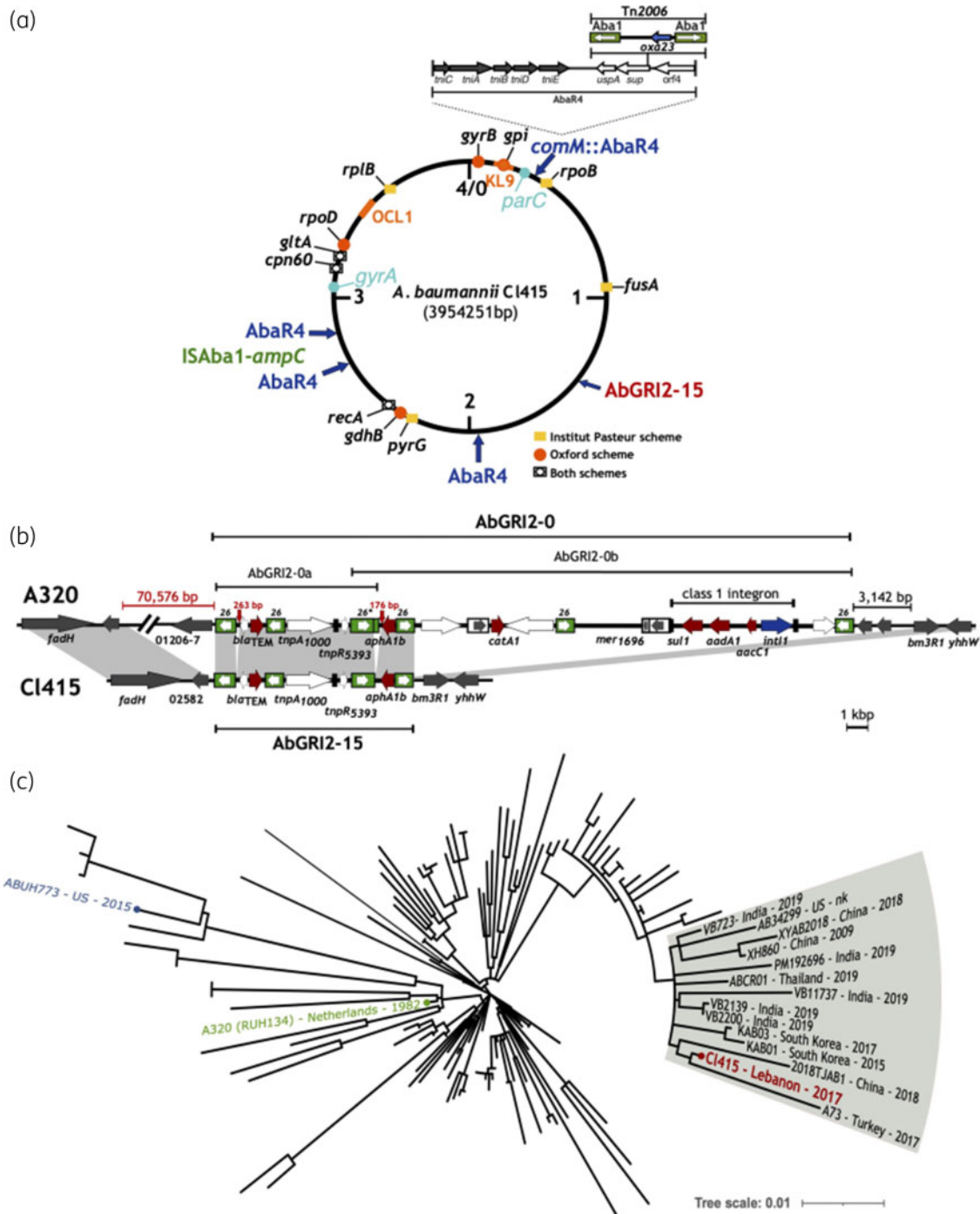
#### Cl415 contains a novel variant of the AbGRI2 antibiotic resistance island

The *bla*<sub>TEM</sub> and *aphA1* genes are in a novel variant of AbGRI2, here called AbGRI2-15 (Figure 1b). AbGRI2-15 is a 9680 bp IS26-flanked structure that has lost several segments, when compared with the ancestral structure AbGRI1-0,<sup>11</sup> due to multiple IS26-mediated deletion/recombination events, which is a well-known property of IS26.<sup>6,33</sup> AbGRI2-15 includes two internal deletions (263 and 176 bp) and two external deletions on either side, which extend to the chromosome (Figure 1b). AbGRI2-15 is a novel structure as it could not be found in any other strain in GenBank.

#### Cl415 is a newly diverged GC2 strain

To place Cl415 in the GC2 phylogeny, and examine its relationship to ABUH773, a whole-genome phylogenetic tree of all complete GC2 genomes was constructed (139 genomes as of April 2021; Table S2). Cl415 clusters with a subclade of GC2 strains (and not with ABUH773) from diverse geographical regions, all recovered after 2017 (Figure 1c). Analysis of SNPs shows that members of the Cl415 clade differ by 19–50 SNPs while they differ from the remaining GC2 strains by 50–120 SNPs (Table S3).

Given that Cl415 and ABUH773, which also contains an AbaR4 in *comM*, belong to different clades (Figure 1c), this indicates that AbaR4 has targeted the *comM* of GC2 strains on at least two occasions. Sequence analysis showed that all other members of the Cl415 subclade have an AbGRI1 island in their *comM* (data not shown). In addition, none of the members of this subclade contain AbGRI2-15, indicating that even though all these strains are closely related, Cl415 has diverged recently by acquisition of AbaR4 in *comM*, AbaR4 transposition to three additional locations and deletions in AbGRI2-15, giving rise to a new strain. This raises concern if a strain such as Cl415 spreads internationally, given that it belongs to the most successful clone of *A. baumannii* and is resistant to very high levels of carbapenems. Currently there are not sufficient genome sequence data available from Lebanon, making it difficult



**Figure 1.** Circular map of the Cl415 chromosome (a), structure of AbGRI2-15 compared with AbGRI2-0 (b) and phylogenetic relationship of Cl415 to other complete ST2 genomes (c). In (a), genes used in MLST schemes are indicated using different colours. The chromosomal positions of *AbaR4* copies are shown using blue arrows. The structure of the *AbaR4* carbenicillin resistance island is shown next to the blue arrow that indicates the *comM::AbaR4* structure. In (b), horizontal arrows represent the extent and direction of genes, with red arrows showing antibiotic resistance genes, grey chromosomal genes, and those coloured white indicating genes with unknown function. Green filled boxes indicate IS26 copies. Shades of grey indicate regions with 99.9%–100% identity. The extent of AbGRI2-0, AbGRI2-0a and AbGRI2-0b are shown above using horizontal lines. In (c), Cl415 subclade of GC2 is shown using a grey background. ABUH773 (also containing *AbaR4* in *comM*) and A320 (RUH134), the GC2 reference strain, which was also used as reference to construct the phylogenetic tree, are also indicated. Tree scale is also shown. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.



**Table 1.** Properties of strains containing *AbaR4* in the *comM* gene

<i>Acinetobacter</i> species	Strain	ST <sub>IP</sub> <sup>a</sup>	Country	Date	Source	Accession no.
<i>A. baumannii</i>	Cl415 <sup>b</sup>	2	Lebanon	2017	Blood	CP071764
<i>A. baumannii</i>	ABUH773 <sup>b</sup>	2	USA	2015	NK	CP035049
<i>A. baumannii</i>	D36 <sup>c</sup>	81	Australia	2008	Wound	CP012952
<i>A. baumannii</i>	PM193665	10	India	2019	Pus	CP050415
<i>A. baumannii</i>	PM194188	10	India	2019	BAL	CP050425
<i>A. baumannii</i>	Ab04-mff	10	Canada	2012	Blood	CP012006
<i>A. baumannii</i>	CIAT758	10	India	2018	Blood	CP038500
<i>A. baumannii</i>	B11911	149	India	2014	Blood	CP021345
<i>A. baumannii</i>	KSK19	622	India	2020	RS	CP072295
<i>A. baumannii</i>	KSK20	622	India	2020	RS	CP072300
<i>A. baumannii</i>	KSK18	622	India	2020	RS	CP072290
<i>A. baumannii</i>	KSK7	622	India	2020	RS	CP072275
<i>A. baumannii</i>	KSK10	622	India	2020	RS	CP072280
<i>A. baumannii</i>	KSK6	622	India	2020	RS	CP072270
<i>A. baumannii</i>	VB16141	622	India	2019	Blood	CP040050
<i>A. baumannii</i>	IOMTU 433	622	Nepal	2013	NK	AP014649
<i>A. baumannii</i>	VB35179	1512	India	2018	Blood	CP040053
<i>A. baumannii</i>	VB35435	575	India	2018	Blood	CP040056
<i>A. baumannii</i>	VB82	25	India	2019	Blood	CP050385
<i>A. baumannii</i>	HWBA8	25	South Korea	2013	Sputum	CP020597
<i>A. baumannii</i>	P7774	25	India	2017	Pus	CP040259
<i>A. baumannii</i>	SD	—	USA	2018	Clinical	CP064292
<i>A. baumannii</i>	NIPH17_0019	571	Cambodia	2017	Blood	AP024415
<i>A. nosocomialis</i>	SSA3	433	South Korea	2013	Blood	CP020588
<i>A. seifertii</i>	AS58	553	Taiwan	2010–7	Blood	CP061598
<i>A. nosocomialis</i>	2010N17-248	410	Taiwan	2010	NK	CP033572
<i>A. pittii</i>	2014N05-125	1611	Taiwan	2014	NK	CP033525

NK, not known; RS, respiratory sample.

<sup>a</sup>Institut Pasteur scheme.

<sup>b</sup>GC2.

<sup>c</sup>GC1.

to track the local spread (and international spread) of Cl415 or its progenies. This calls for more genome sequence data from Lebanon as well as from diverse geographical regions.

## Conclusions

Cl415 contains AbGRI2-15, which is a novel AbGRI2 island that contains *aphA1* and *bla*<sub>TEM</sub>. Cl415 has become resistant to high levels of carbapenems by acquiring four *AbaR4* copies, one of which is in *comM* replacing the AbGRI1 island. Multiplication of *AbaR4* is likely to have occurred in response to high carbapenem selective pressure, highlighting the ability to rapidly develop resistance to high levels of carbapenems. Cl415, recovered in Lebanon, belongs to a clade that contains several strains from different geographical places, but Cl415 has recently diverged by the acquisition of *AbaR4* and loss of multiple AbGRI2 segments.

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## Transparency declarations

None to declare.

## Supplementary data

Tables S1 to S3 are available as [Supplementary data](#) at JAC Online.

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