Genetic environment of the KPC gene in Acinetobacter baumannii ST2 clone from Puerto Rico and genomic insights into its drug resistance

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INTRODUCTION

Acinetobacter baumannii is a Gram-negative, non-fermentative opportunistic pathogen, associated with nosocomial pneumonia, endocarditis, meningitis and bloodstream and urinary tract infections. It has simple growth requirements, is able to survive in dry and moist environments and can tolerate a wide range of pH and temperatures (Getchell-White et al., 1989). This, together with antimicrobial resistance factors, has contributed to its successful adaptation to the hospital environment (Lockhart et al., 2007). Antibiotic resistance in A. baumannii is due to a combination of several factors including low membrane permeability, mutation in its chromosomal genes, overexpression of efflux pumps and acquisition of mobile resistance genes from other organisms (Manchanda et al., 2010).

Carbapenems are considered the last-resort antibiotics to treat infections caused by multidrug-resistant Gram-negative bacilli. The Klebsiella pneumoniae carbapenemase (KPC) enzyme hydrolyses β-lactam antibiotics including the carbapenems. KPC has been detected worldwide in Enterobacteriaceae and Pseudomonas aeruginosa isolates associated with transposon Tn4401 commonly located in plasmids. Acinetobacter baumannii has become an important multidrug-resistant nosocomial pathogen. KPC-producing A. baumannii has been reported to date only in Puerto Rico. The objective of this study was to determine the whole genomic sequence of a KPC-producing A. baumannii in order to (i) define its allelic diversity, (ii) identify the location and genetic environment of the bla_{KPC} and (iii) detect additional mechanisms of antimicrobial resistance. Next-generation sequencing, Southern blot, PFGE, multilocus sequence typing and bioinformatics analysis were performed. The organism was assigned to the international ST2 clone. The bla_{KPC-2} was identified on a novel truncated version of Tn4401e (tentatively named Tn4401h), located in the chromosome within an IncA/C plasmid fragment derived from an Enterobacteriaceae, probably owing to insertion sequence IS26. A chromosomally located truncated Tn1 transposon harbouring a bla_{TEM-1} was found in a novel genetic environment within an antimicrobial resistance cluster. Additional resistance mechanisms included efflux pumps, non-β-lactam antibiotic inactivating enzymes within and outside a resistance island, two class 1 integrons, In439 and the novel In1252, as well as mutations in the topoisomerase and DNA gyrase genes which confer resistance to quinolones. The presence of the bla_{KPC} in an already globally disseminated A. baumannii ST2 presents a serious threat of further dissemination.

Abbreviations: CHDL, carbapenem-hydrolysing β-lactamase; CLSI, Clinical and Laboratory Standards Institute; IS, insertion sequence; KPC, Klebsiella pneumoniae carbapenemase; MBL, metallo-β-lactamase; MLST, multilocus sequence typing; MDR, multidrug resistance; RIR, right inverted repeat.

The GenBank accession numbers for the draft genome of Acinetobacter baumannii M3AC14-8 sequence are LDDY00000001 to LDDY00000030.

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types of carbapenemases, but the presence of insertion sequences (IS) upstream of CHDL genes can provide additional promoters leading to gene overexpression (Turton et al., 2006).

*Klebsiella pneumoniae* carbapenemase (KPC) also renders bacteria resistant to the β-lactam antibiotics, including the carbapenems (Yigit et al., 2001). The KPC gene has been detected worldwide in *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates (Nordmann et al., 2009), in which it is located in transposon Tn4401 (isoforms a to g) in plasmids of different sizes and incompatibility groups (Chmelnitsky et al., 2014). In 2009, our laboratory reported, for the first time, the presence of the KPC gene in *A. baumannii*, and to our knowledge, KPC-producing *A. baumannii* has been reported only in Puerto Rico (Robledo et al., 2010; Martínez et al., 2014).

The whole-genome sequence has been a useful tool to understand the evolution of *A. baumannii* and movement of its resistance genes and virulence factors. *A. baumannii* is a naturally transformant bacterium which does not discriminate between itself and foreign DNA, hence its great genomic plasticity (Zarrilli et al., 2013). Whole-genome sequence studies have demonstrated that *A. baumannii* strains have an extensive gene variation between phylogenetically closely related isolates and even from those obtained from the same patient (Wright et al., 2014).

The objective of this study was to determine the whole-genome sequence of a KPC-producing *A. baumannii* M3AC14-8 in order to (i) define the isolate’s allelic diversity, (ii) identify the location of the KPC gene and its genetic environment and (iii) detect additional mechanisms of antimicrobial resistance.

**METHODS**

**Bacterial strains.** In 2014, four multidrug-resistant KPC-producing *A. baumannii* strains were identified in a single institution from different patients during a 4-month period. They were sent to our laboratory together with their corresponding susceptibility reports and basic epidemiological information. The four isolates were primarily identified in the clinical microbiology laboratory by the VITEK 2 system, and the identification of the isolates as *A. baumannii* was confirmed in our laboratory by biochemical tests (cytochrome c oxidase test and Triple Sugar Iron slants), PCR detection of the *blaOXA-51* gene, as described by Woodford et al. (2006), and sequencing of the 16S rDNA gene, as described by Mannoni et al. (2003). PCR screening with family-specific β-lactamase primers for TEM, KPC and OXA carbapenemases was performed as previously described by Moland et al. (2006) and Woodford et al., 2006) using bacterial control strains isolated in our laboratory from previous surveillance studies (Robledo et al., 2010; Martínez et al., 2012). No attempts were made to evaluate patients’ therapies or clinical outcomes.

**Determination of genetic relatedness by PFGE.** Genetic relatedness between the *A. baumannii* isolates was determined by PFGE, as described by Durmus et al. (2009) and Goering et al. (2010). DNA samples were prepared by in situ lysis of cells encased in agarose and digested with Apal. PFGE was performed using a CHEF DR III System with the following conditions: switching from 5–30 s for 20 h at 6 V cm⁻¹, 14°C and 120° angle.

**Multilocus sequence typing.** Multilocus sequence typing (MLST) was performed using the Institut Pasteur MLST database available online (http://www.pasteur.fr/mlst).

**Next-generation sequencing and bioinformatics analysis.** Whole-genome sequencing was commercially performed by GeneWiz, using an Illumina MiSeq 2 × 150 bp paired-end configuration. *De novo* assembly and genome annotation were performed using the Pittsburgh Supercomputing Center Blacklight supercomputer system (Townes et al., 2014) (manuscript in press). ORFs were predicted using Prodigal (version 2.60) (Hyatt et al., 2010). Isos and resistance genes were identified with IS Finder Web site (Sigueri, 2006) and RES finder (Zankari et al., 2012), respectively. Prophage sequences within the bacterial genome were identified using PHAST server (Zhou et al., 2011). Whole-genome multiple sequence alignment was performed using Mauve (Darling et al., 2004). The genomes were submitted to INTEGRALL database for integrion number assignment (Moura et al., 2009).

**Southern blot.** Total DNA plugs were digested with I-CeuI enzyme or S1 nuclease, as described by Liu et al. (1993) and Barton et al. (1995), respectively. Southern blot hybridization was carried out using DIG Easy Hyb Granules using two probes: the 16S rDNA gene to distinguish between chromosomal and plasmid DNA and an internal probe for the KPC gene. Hybridization and development were performed according to the DIG application manual (Roche Applied Science).

**Nucleotide accession number.** The 30 scaffolds of the draft genome sequence of *A. baumannii* M3AC14-8 were submitted to GenBank Genome database and can be found under the accession numbers LDDY00000001 to LDDY00000030.

**RESULTS AND DISCUSSION**

**Patient data and clinical characteristics**

Table 1 shows the clinical characteristics and antimicrobial susceptibilities of the four *A. baumannii* isolates. The samples were collected from skin and soft tissue, respiratory tract and urine between April and August 2014. The patients’ median age was 54 years (range, 52–90). No differences were observed between gender and hospital ward. The two samples isolated from the general ward were susceptible only to tigecycline, while the two from the intensive care unit were resistant to all tested antibiotics.

**Strain typification**

The four *A. baumannii* clinical isolates were positive for OXA-51-like, KPC and TEM genes. Since PFGE-Apal analysis showed that they had identical pulsotypes (data not shown), only M3AC14-8 strain was selected for further characterization. Using the Institut Pasteur MLST database, the M3AC14-8 strain was assigned to the worldwide-disseminated sequence type ST2. Although *A. baumannii* ST2 clone has been previously associated with the production of CHDLs (OXA-23, OXA-24/40 and OXA-58) and the VIM MBLs, this is the first report of the identification of *blaKPC-2* in this international clone (Bakour et al., 2014; Giannouli et al., 2010; Mammina et al., 2011). Multiple
Table 1. Clinical information and antimicrobial susceptibility profile of KPC-producing A. baumannii isolates

GW, general ward; ICU, intensive care unit; AMK, amikacin; AMS, ampicillin-sulbactam; FEP, cefepime; CIP, ciprofloxacin; MEM, meropenem; TGC, tigecycline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Source</th>
<th>Hospital ward</th>
<th>Collection date</th>
<th>MIC of antibiotic tested (µg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3AC14-7</td>
<td>M</td>
<td>52</td>
<td>Respiratory</td>
<td>ICU</td>
<td>4/11/2014</td>
<td>≥64 ≥32 ≥64 ≥4 ≥16 ≥8</td>
</tr>
<tr>
<td>M3AC14-8</td>
<td>F</td>
<td>67</td>
<td>Skin</td>
<td>ICU</td>
<td>4/11/2014</td>
<td>≥64 ≥32 ≥64 ≥4 ≥16 ≥8</td>
</tr>
<tr>
<td>M3AC14-16</td>
<td>M</td>
<td>90</td>
<td>Respiratory</td>
<td>GW</td>
<td>6/3/2014</td>
<td>≥64 ≥32 ≥64 ≥4 ≥16 2</td>
</tr>
<tr>
<td>M3AC14-32</td>
<td>F</td>
<td>63</td>
<td>Urine</td>
<td>GW</td>
<td>8/13/2014</td>
<td>≥64 ≥32 ≥64 ≥4 ≥16 2</td>
</tr>
</tbody>
</table>

Fig. 1 shows the schematic representation of the 89 kb DNA fragment (scaffold 8) harbouring the Tn\(^{4401}\)h. This scaffold has a 54 % GC content and 129 ORFs, distributed as 48 hypothetical proteins and 81 proteins with known functions. Among the identified proteins are a partial copy of IS26 at the beginning of the scaffold (nt 1–361), an A/C replicase gene, an ISKpn6, a non-functional endonuclease subunit M and a truncated version of a Tn6187-like transposon. The scaffold ends with a class 1 integron. Additional copies of IS26 were found in other scaffolds of the draft genome sequence (three complete and eight incomplete copies at the end of the scaffolds), which made it impossible to assemble the complete genome sequence. Recent studies have demonstrated that IS26 has an important role in the creation, dissemination and reorganization of antimicrobial resistance gene clusters in Enterobacteriaceae plasmids, including IncA/C plasmids, and in A. baumannii isolates (He et al., 2015; Nigro et al., 2013; Lean et al., 2015). IncA/C plasmids are large conjugative plasmid from Enterobacteriaceae with a broad host range and different hotspot sites for the integration of multidrug resistance (MDR) genes (Fricke et al., 2009). The IncA/C plasmid has been previously reported in A. baumannii only by Zhang et al. (2014) in a survey of Gram-negative bacilli clinical isolates from China; however it is unknown if the plasmid was integrated into the chromosome since they used total DNA extraction to detect the replicase genes by PCR.

The IncA/C plasmid fragment in M3AC14-8 strain is closely related to the KPC-harbouring plasmid pBWH-C7-KPC from a K. pneumoniae isolated in Massachusetts, USA (BioSample accession no. SAMN03657219; Pecora et al., 2015) and the non-KPC-producing pR55 plasmid from a K. pneumoniae isolated in 1969 in France (accession no. JQ010984; Doublet et al., 2012). Similar to plasmid pBWH-C7-KPC, M3AC14-8 strain contained 37 of the 42 proteins encoded by the enterophage P2 (accession no. AF063097) inserted at the same position into a hypothetical protein; however, plasmid pR55 does not contain the P2 phage (plasmid comparison not shown).

Fig. 2 shows a schematic representation of the genetic background surrounding the Tn\(^{4401}\)h in the KPC-producing M3AC14-8 strain (Fig. 2a) and its comparison with the KPC-harbouring plasmid pBWH-C7-KPC (Fig. 2b) and the non-KPC-plasmid pR55 (Fig. 2c), obtained from doublet et al. (2009). The KPC-harbouring plasmid pBWH-C7-KPC includes a truncated restriction endonuclease subunit M and a truncated DNA replication protein flanking the Tn\(^{4401}\)h, followed by a truncated version of a Tn6187-like transposon (Fig. 2a). Tn\(^{4401}\)h retained the transposon right inverted repeat (RIR) sequence (blue triangle in Fig. 2a) and 14/15 nucleotides of the ISKpn7 RIR (5′-TGTTAG-CAGCAGTGT-3′, mismatched nucleotide is underlined, yellow rectangle in Fig. 2a), as described by Naas et al. (2008). Upstream of the RIR of the Tn\(^{4401}\)h in M3AC14-8 strain, a 5 bp DNA sequence (GGGAA) and the 5′ end fragment of a DNA replication protein (accession no. WP_
enzymes and bla position cis integrated into the Tn have caused the inversion of the truncated version of IS identified in clinical isolates of K. pneumoniae. As previously mentioned, similar to M3AC14-8 strain, et al. (2015) before the plasmid was integrated into the A. baumannii chromosome. The aacA42 gene cassette in the novel integron In1252 has only been described twice in the literature associated with blag35 type enzymes and blatxa-2 in two integrons, In647 (accession no. GQ337064) and In724 (accession no. JN596280) identified in clinical isolates of P. aeruginosa from Spain (Viedma et al., 2009) and Enterobacteriaceae from Mexico (Barrios et al., 2012), respectively.

As previously mentioned, similar to M3AC14-8 strain, K. pneumoniae pBWH-C7-KPC plasmid has a Tn6187-like transposon followed by Tn4401h but in an inverted orientation (Fig. 2b), inserted into the same DNA replication protein, and this insertion generates the same 5 bp duplication site (GGGAA) observed in M3AC14-8 strain (Fig. 2a). Plasmid pBWH-C7-KPC, in addition, has the same gene cassette array (5’CS-aadB, aacA42-3’CS) in the class 1 integron as M3AC14-8 strain; however, plasmid pBWH-C7-KPC does not have an IS5075 copy upstream to the Tn21 mercury resistance module. DNA sequence analysis of K. pneumoniae pBWH-C7-KPC plasmid demonstrated a 128 bp localized in nt 59530–59657 identical to the 128 bp of ISKpn7 (including the 15 bp of the RIR) localized in nt 172436–172563. Since the DNA fragment between them (which is mostly the tra genes responsible for plasmid conjugation) present in pBWH-C7-KPC plasmid, but not found in M3AC14-8 strain, we speculate that Tn4401h may have resulted from a homologous recombination event between the 128 bp regions after the inversion event of Tn4401 and the truncated version of Tn6187-like transposon. The locations of these features may suggest that these two KPC-carrying IncA/C plasmids have evolved from a common ancestor through multiple genetic events.

**blaTEM-1 and its genetic environment**

Fig. 3 shows a schematic representation of the immediate environment surrounding the blaTEM-1 in M3AC14-8 strain (Fig. 3a) located upstream of an antimicrobial resistance cluster and its comparison with the genetic structure of A. baumannii HX386 (accession no. CP010779) (Fig. 3b) and from A. baumannii MDR-ZJ06 plasmid (accession no. CP001938) (Fig. 3c). As shown in Fig. 3(a), the chromosomally located blaTEM-1 in M3AC14-8 strain (scaffold 2) was identified in a novel 7.6 kb genetic environment, followed by a 19.9 kb DNA fragment that contains the antimicrobial resistance cluster. The order of the genes in this novel 7.6 kb DNA fragment consists of an IS26, a truncated version of a Tn1 harbouring blaTEM-1 and tnpR, an IS4321L, followed by the tnpA and tnpR genes of a truncated version of Tn1696 transposon (Fig. 3a). The 7.6 kb DNA fragment was inserted upstream of a 19.9 kb antimicrobial resistance cluster that is similar to that found in A. baumannii HX386, sharing 98% of its DNA sequence (Fig. 3b), and in A. baumannii MDR-ZJ06 plasmid, sharing 91% of its DNA sequence (Fig. 3c). The M3AC14-8 cluster...
includes In439 class I integron, five different IS elements (two IS26 copies, one copy of a truncated ISAba24 and one copy each of ISCR, ISEc28 and ISEC29) and the antimicrobial resistance genes for macrolides (\textit{murE} and \textit{mphE}) and the 16S rDNA methyltransferase (\textit{armA}) that confers resistance to aminoglycosides (Fig. 3a). The gene cassette of the In439 class I integron (\textit{5\'CS-aacA4'-8-catB8-aadA1a-aadA1a-3\'CS}) in \textit{M3AC14-8} strain is highly similar to \textit{A. baumannii} HX386, except that, in \textit{M3AC14-8} strain, the \textit{intI1} gene is disrupted by 415 bp which includes a duplication of 219 bp of the \textit{intI1} gene and 196 bp that belongs to an IS26 fragment. The In439 confers resistance to aminoglycosides (\textit{aacA4'}-8 and \textit{aadA1a}) and to chloramphenicol (\textit{catB8}). \textit{A. baumannii} MDR-ZJ06 plasmid contains a different class 1 integron, In561 (\textit{5\'CS-aacC1-gcuP-gcuQ-aadA1a-3\'CS}), as shown in Fig. 3(c). \textit{A. baumannii} HX386 and \textit{A. baumannii} MDR-ZJ06 plasmid share 93\% of their DNA sequence; the only difference is that the class 1 integron is In439 in HX386 strain and In561 in MDR-ZJ06 plasmid and the presence of a third IS26 copy in \textit{A. baumannii} HX386 (Fig. 3b, c). Genomic analysis suggests that, in \textit{A. baumannii} HX386, the chromosomal integration of the \textit{A. baumannii} MDR-ZJ06 plasmid was mediated by IS26, and this integration generated the third copy of IS26 and an 8 bp duplication (AGGATGAG), flanking the first and third copies of IS26 (Fig. 3b). Data analysis suggests that, in \textit{M3AC14-8} strain, the IS26 also had a role in the plasmid integration and remodelling of the DNA, since the same 8 bp
duplication (AGGATGAG) was found at the left side of the IS26 upstream of blaTEM-1. An intramolecular transposition in trans of IS26 was responsible for the DNA inversion and the truncated version of the IS26 in M3AC14-8 strain when compared with A. baumannii HX386. IS26 may also have been involved in the integration of a 7.6 kb DNA fragment that contains the truncated Tn1 harboring the blaTEM-1 upstream of the 19.9 kb DNA fragment.

**Other resistance mechanisms**

Additional mechanisms of antibiotic resistance to non-β-lactam antibiotics could be inferred from the antimicrobial susceptibility results and bioinformatics analysis. The presence of an ISAb1 element adjacent to the ampC cephalosporinase (blaADC-25) gene was identified, which might result in an overexpression of the gene (Héritier et al., 2006). The OXA-51 carbapenemase variant present in M3AC14-8 strain was blaOXA-66, but no IS element was found located upstream of the gene. A partially sequenced resistance island (scaffolds 18 and 23), similar to AbaR22 from A. baumannii MDR-ZJ06 plasmid, was found interrupting the comM gene in M3AC14-8 strain. The resistance island includes resistance genes for streptomycin (strA and strB) and the gene that codes for the tet(B) efflux pump which confers resistance to tetracyclines. The following additional efflux pump systems were identified outside the resistance island: AdeABC, AdeIJK, AbeM and AbeS. Amino acid
variations associated with overexpression of the AdeABC efflux pump in the two-component regulatory system adeSR were identified, adeS and adeR. The amino acid variation N268F1 was identified in adeS (Hornsey et al., 2011); and the V120L, L136I variation, in adeR (Ardebili et al., 2014). Mutations encoding amino acid substitutions associated with quinolone resistance were identified in DNA gyrase (gyrA) and topoisomerase (parC), the S83L and S80L substitutions, respectively (Sun et al., 2015).

Plasmid characterization

Two complete and circularized plasmids were found in M3AC14-8 strain. Both plasmids, p1M3AC14-8 and p2M3AC14-8, exhibited a replicase gene and two mobilization genes but lacked the genes involved in partition and conjugation. The plasmid p1M3AC14-8 is 5541 bp with a G+C content of 35 % and is very similar to a 5644 bp A. baumannii AYE plasmid, p1ABAYE (accession no. CU459137), sharing 91 % of their DNA sequence (with an expected value of 0). As expected, the replicase gene, repA (316 aa), identified in plasmid p1M3AC14-8, is identical to the replicase gene of p1ABAYE and belongs to the homology group 11 (Gr11) identified by A. baumannii PCR-based replicon typing scheme described by Bertini et al. (2010). The plasmid consists of six ORFs, two hypothetical proteins, a mobilization protein MobL, a putative mobilization protein MobS and a putative toxin–antitoxin system.

The second plasmid, p2M3AC14-8, was determined to be 18043 bp with an average G+C percentage of 35. Blast against NCBI database shows that p2M3AC14-8 is a novel plasmid sharing only 38 % of the DNA sequence (with 99 % identity and an expected value of 0) with a 10679 bp p2M3AC14-8 strain. Both plasmids, p1M3AC14-8 and p2M3AC14-8, exhibited a replicase gene and two mobilization proteins, a mobilization protein MobS and a putative toxin–antitoxin system.

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References


