Characterization of plasmids carrying the $\text{bla}_{\text{OXA-24/40}}$ carbapenemase gene and the genes encoding the AbkA/AbkB proteins of a toxin/antitoxin system

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Background: Carbapenem-resistant Acinetobacter baumannii (CRAb) is a major source of nosocomial infections in Spain associated with the production of OXA-58-like or OXA-24/40-like $\beta$-lactamase enzymes. We analysed the plasmids carrying the $\text{bla}_{\text{OXA-24/40}}$-like gene in CRAb isolates obtained a decade apart.

Methods: The presence of $\beta$-lactamases was screened for by PCR (metallo-$\beta$-lactamases, carbapenem-hydrolysing class D $\beta$-lactamases, GES and KPC) in 101 CRAb isolates obtained in two multicentre studies (GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010; n = 493 Acinetobacter spp). We analysed the distribution and characterization of the plasmids carrying the $\text{bla}_{\text{OXA-24/40}}$-like gene and sequenced two plasmids, AbATCC223p (2000) and AbATCC329p (2010) from A. baumannii ATCC 17978 transformants.

Results: Acquisition of the $\text{bla}_{\text{OXA-24/40}}$-like gene was the main mechanism underlying resistance to carbapenems (48.7% in 2000 compared with 51.6% in 2010). This gene was mainly isolated in ST2 A. baumannii strains in both studies, although some novel STs (ST79 and ST80) appeared in 2010. The gene was located in plasmids (8–12 kbp) associated with the repAci2 or repAci2/repGR12 types. The sequences of AbATCC223p (8840 bp) and AbATCC329p (8842 bp) plasmids were similar, particularly regarding the presence of the genes encoding the AbkA/AbkB proteins associated with the toxin/antitoxin system. Moreover, the abkA/abkB gene sequences (>96% identity) were also located in plasmids harbouring the $\text{bla}_{\text{OXA-58}}$-like gene.

Conclusions: The action of OXA-24/40 and OXA-58 $\beta$-lactamase-like enzymes represents the main mechanism underlying resistance to carbapenems in Spain in the last decade. AbkA/AbkB proteins in the toxin/antitoxin system may be involved in the successful dissemination of plasmids carrying the $\text{bla}_{\text{OXA-24/40}}$-like gene, and probably also the $\text{bla}_{\text{OXA-58}}$-like gene, thus contributing to the plasmid stability.

Keywords: Acinetobacter baumannii, A. baumannii, $\text{bla}_{\text{OXA-24/40}}$-like

Introduction

Carbapenem-resistant Acinetobacter baumannii (CRAB) is currently a major source of nosocomial infections and is considered a highly successful human pathogen. Among the different mechanisms associated with carbapenem resistance in A. baumannii, the production of acquired carbapenem-hydrolysing class D $\beta$-lactamases (CHDLs) and class B metallo-$\beta$-lactamases (MBLs) has been widely studied. Three different classes of CHDLs acquired in CRAB have so far been reported in Spain: OXA-24/40-like, OXA-58-like and OXA-23. However, OXA-24/40-like and OXA-58-like have been the predominant CHDLs found in the Iberian Peninsula.
In the present study we investigated the distribution and clonal relatedness of CRAB strains carrying the bla\textsubscript{OXA-24/40}-like gene from two Spanish multicentre studies (GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010), as well as the plasmids carrying it.

**Materials and methods**

**Bacterial strains, susceptibility testing and carbapenemase detection**

In total, 493 Acinetobacter spp. isolates were analysed in the two multicentre studies (GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010). Identification was made to species level by MALDI-TOF and amplified ribosomal DNA restriction analysis. Fernández-Cuenca et al. studied the differences in the susceptibility of all isolates obtained in the two studies (GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010) to a wide range of antimicrobial agents and found an increase in resistance to carbapenem of up to 83%. In a study of the clonal relationships of all the isolates using REP-PCR, PFGE and MLST, Villar et al. found that the overall increase in carbapenem resistance appeared to be related to the development of resistance in strains belonging to the dominant clonal groups such as ST2. A total of 101 clonally unrelated CRAB isolates identified by REP/PFGE (39 compared with 62 strains from GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010, respectively) were selected to study the detection of β-lactamases by PCR screening of the genes encoding MBLs, CHDLs, GES and KPC.

**Plasmid analysis**

The representative isolates from the REP/PFGE-groups carrying a bla\textsubscript{OXA-24/40}-like gene (n = 48) from both GEIH/REIPI-Ab studies were selected for subsequent plasmid characterization. In order to homogenize the genetic background of the strains, these plasmids were introduced into the A. baumannii ATCC 17978 by electroporation. Plasmid profiles were obtained by restriction fragment analysis and replicon typing according to the literature.

**Next generation sequencing and bioinformatic analysis of the A. baumannii ATCC 17978 strain with plasmids harbouring the bla\textsubscript{OXA-24/40}-like gene**

A. baumannii ATCC 17978 transformant strains (AbATCC223s and AbATCC329s) with the same plasmid restriction profile (P3), the Ab223 clinical isolate from GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010, respectively, were selected to study the detection of β-lactamases by PCR screening of the genes encoding MBLs, CHDLs, GES and KPC.

**Characterization of plasmids carrying the bla\textsubscript{OXA-24/40}-like gene**

All plasmids from A. baumannii ATCC 17978 transformant strains bearing the bla\textsubscript{OXA-24/40}-like gene ranged between 8 and 12 kbp in size. Only one isolate from GEIH/REIPI-Ab-2000 contained a non-typeable plasmid harbouring the bla\textsubscript{OXA-24/40}-like gene. The plasmid types carrying the bla\textsubscript{OXA-24/40}-like gene most frequently observed in both studies were P1 and P3, containing repAcI2/repGR12 or repAcI2 sequences and indistinctively associated with different STs (Table S1, available as Supplementary data at JAC Online).

**Genome of the ATCC 17978 transformant strains (AbATCC2329s/AbATCC223s) with AbATCC2323p and AbATCC329p**

The in silico results were as follows: (i) 178 782 reads were obtained from a sequencing process of AbATCC329s (2010) with a mean length of 423 nucleotides, whereas 187 192 reads were obtained from a sequencing process of AbATCC223s (2000) with a mean length of 469 nucleotides; (ii) ‘de novo’ assembly gave rise to 77 contigs for AbATCC329s (2010) and 186 contigs for AbATCC223s (2000), for a total of 4.02 Mbp assembled nucleotides in the former and 4.02 Mbp assembled nucleotides in the latter; and (iii) ORF prediction gave rise to 3877 predicted ORFs for AbATCC329s (2010), whereas 38 325 ORFs were predicted for AbATCC223s (2000). Plasmids AbATCC223p and AbATCC329p (P3 by plasmid restriction profile) were both very similar (99% similarity) to PMMCU3 plasmid harbouring the bla\textsubscript{OXA-24/40}-like gene (Table 1). These plasmids displayed 11 ORFs in which the possible role of the proteins (ORFs 6, 9, 10 and 11) remains unknown.

**AbkA/AbkB proteins from toxin/antitoxin systems**

The results of the Glimmer3 (version 3.02) analysis showed that these genes (abkA/abkB) belonged to toxin/antitoxin systems. Moreover, these proteins displayed high homology (97% identity) with other proteins from these systems in species of Acinetobacter as well as in other bacteria such as Escherichia coli, Salmonella enterica and Citrobacter freundii (57% identity). Indeed these proteins belong to family DUF4415 (anti-toxin component) and to family DUF497 (toxin component). Genomic features used for discrimination of the toxin/antitoxin system were found in the abkAB operon: (i) in two genes the

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‘dormant guard’ role is fulfilled by the combined presence of a toxin and a protective protein; and (ii) toxin/antitoxin pairs are encoded by genes forming an operon (the spacer seldom extends beyond 30 nucleotides) and a small overlap (usually 1–20 nucleotides) (see Table 1).

The presence of abkA/abkB genes as well as other genes (such as that encoding septicolysin, a putative virulence factor) in AbATCC223p and AbATCC329p was confirmed by PCR in all of the clinical strains harbouring blaOXA-24/40-like genes. Furthermore, the abkA/abkB genes were found to be conserved in several plasmids carrying OXA-72 β-lactamase and OXA-58 β-lactamase (Table S2, available as Supplementary data at JAC Online). The presence of the abkA/abkB genes (ORF positions 3661–3948/3941–4249) in the p2-ABST25 plasmid located in strain 4190 (ST25) (the genome of which was recently published19) was of particular interest.
The action of the OXA-24/40-like enzyme remains the main mechanism of carbapenem resistance, although there has been a slight increase in the percentage of OXA-58-like producing isolates. Only one A. baumannii clinical OXA-23-like producing strain has been detected, in a sample from a hospital in Palma de Mallorca.26

On the other hand the present results showed that the presence of the bla_{OXA-24/40} gene in Spain during the last decade is confined to a few genetically related outbreak clones mostly belonging to ST72.26 However, attention should be paid to the emergence in 2010 of novel STs not detected in the previous study, such as ST79 and ST80.

The bla_{OXA-24/40} -like gene is carried in a small number of plasmid types (8–12 kbp) associated with rep-types rep{Aci}2 or rep{Aci}2 in combination with repGR12, some of which are present in both studies, and hence there were no significant differences between the populations of plasmids found in 2000 and 2010. In addition, all the plasmids in this study (GEIH/REIPI-Ab-2000 and GEIH/REIPI-Ab-2010) contained two proteins (Abk{A}/Abk{B}) belonging to a toxin/antitoxin system that is similar to other toxin/antitoxin systems from several bacteria. The PMMA2 and PMM CU3 plasmids (which showed a high degree of homology) harbouring the bla_{OXA-24/40} -like gene15 were sequenced in an outbreak of A. baumannii in Spain. However, other plasmids harbouring the bla_{OXA-24/40} -like gene from this outbreak, such as PMM CU1, pMM D and pMM CU2, did not have this system (the latter two plasmids were isolated from a smaller number of clones of A. baumannii during the outbreak).15 Other plasmids carrying the β-lactamases OXA-72 and OXA-58 also had this system.21,22

Plasmid stabilizing toxin/antitoxin systems have been used as examples of selfish DNA in the gene-centred view of evolution. It has been theorized that toxin/antitoxin loci serve only to maintain their own DNA, at the expense of the host organism.23 Other theories propose that these systems have evolved to increase the fitness of plasmids in competition with other plasmids.24 Thus, the toxin/antitoxin system confers an advantage to the host DNA by eliminating competing plasmids in the cell progeny. This theory has been corroborated by computer modelling.25 Toxin/antitoxin systems have been categorized into three types according to the nature of the antitoxin and the composition of toxin/antitoxin operon.26 According to the genomics features, the Abk{A}/Abk{B} system may belong to the type II toxin/antitoxin system (11 type II toxin/antitoxin families have been described in prokaryotes).26

Moreover, molecules that block the normal operation of toxin/antitoxin systems are considered to be a potent target for the development of new antimicrobial agents. A simple method of inhibiting antitoxin binding to toxin may improve the activity of toxins, which may result in bacterial death.26

In conclusion, the results presented here show that the increased resistance to carbapenem in A. baumannii in Spain between 2000 and 2010 is largely due to the clonal spread of a few genetically related clones (mainly ST2, ST79 and ST80) carrying a bla_{OXA-24/40} -like gene located in plasmids (8–12 kbp) that are highly endemic in specific regions. Abk{A}/Abk{B} proteins located on these plasmids belonging to toxin/antitoxin systems may explain the success of these and of the carbapenem resistance in A. baumannii conferred by the bla_{OXA-24/40} -like gene. Moreover, this system has also been located in plasmids harbouring the bla_{OXA-58} -like gene. Further study of the mechanism of action of this toxin/antitoxin system in Acinetobacter spp. is required to identify a possible target for the development of inhibitor agents as new antimicrobial agents.

**Nucleotide sequence accession numbers**

The sequences of AbATCC223p and AbATCC329p plasmids were deposited in GenBank under nucleotide sequence numbers KJ534568 and KJ534569, respectively.

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Transparency declarations
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Supplementary data
Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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