Loss of LPS is involved in the virulence and resistance to colistin of colistin-resistant Acinetobacter nosocomialis mutants selected in vitro

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Objectives: Acinetobacter nosocomialis has increasingly been reported as an opportunistic pathogen causing nosocomial infections. Although it is more susceptible to all antimicrobial agents than Acinetobacter baumannii, MDR clinical isolates have also been described. In addition, several studies have shown a high percentage of resistance to colistin. Therefore, in the present study we investigated the mechanism of resistance to colistin in this microorganism.

Methods: Colistin-resistant strains were selected from the original colistin-susceptible A. nosocomialis strain following multi-step mutant selection. Comparative genomic and proteomic analyses of both colistin-susceptible and colistin-resistant A. nosocomialis strains were performed. In addition, virulence was investigated using the Caenorhabditis elegans assay.

Results: The colistin-resistant mutants selected showed a lower resistance profile for other types of antibacterial agents together with a significant decrease in virulence. The LT50 (i.e. time required to kill 50% of the nematodes) for the colistin-susceptible strain (WT) was 7 days compared with 9 days for the colistin-resistant strain (256) (P<0.0001). In the genomic studies, several mutations were observed in the lpxD genes, leading to the loss of LPS in the colistin-resistant strains. The proteomic studies showed several up- and down-regulated proteins that may be involved in colistin resistance or in a decrease in the resistance profile for several antibiotics.

Conclusions: This study shows that the mechanism of resistance to colistin by A. nosocomialis is mainly associated with the loss of LPS due to mutations in the lpxD gene, although changes in the expression of some proteins cannot be ruled out. In addition, the acquisition of colistin resistance is related to a decrease in virulence.

Introduction

In the Acinetobacter genus, the Acinetobacter group consists of Acinetobacter pittii, Acinetobacter nosocomialis and Acinetobacter baumannii, which are also the most common species isolated in the nosocomial setting. A. baumannii has demonstrated the ability to acquire resistance to all of the antibiotics available on the market (pan-resistance). Resistance to carbapenems has been associated with the acquisition of carbapenemases,1 with some of these carbapenemases, such as OXA-23,2 OXA-583 and NDM-1,4,5 also having been reported in A. nosocomialis.

Polymyxins are the last option in the treatment of infections caused by XDR A. baumannii. To date, most A. baumannii strains are still susceptible to colistin. However, in recent years colistin-resistant A. baumannii clinical isolates have been reported.1,6 Different mechanisms of resistance to colistin in A. baumannii have been elucidated. These mechanisms are related to each other and have some relationship with membrane components. Adams et al.7 observed mutations in the pmrABC operon. PmrAB is a two-component regulatory system that regulates the expression of the pmrC gene, which encodes a phosphoethanolamine transferase enzyme involved in lipid A modification.7–9 The second mechanism of resistance is the complete loss of LPS by mutations in the lpxACD genes, which are involved in lipid A biosynthesis.5,10 In a recent study, Park et al.11 identified and validated six genes that encode PmrAB two-component regulatory
enzymes, PmrC, a glycosyltransferase, a poly-β-1,6-N-acetylglucosamine deacetylase and a putative membrane protein by transcriptomic analysis. They found that all these proteins were associated with either LPS biosynthesis or modification of its electrostatic properties, hence confirming that modification of LPS is one of the principal methods for acquiring colistin resistance.

Several studies have reported a high level of resistance to colistin in A. nosocomialis compared with A. baumannii, ranging from 6.5% to 45.3%. Therefore, the main objective of this study was to determine the mechanism of resistance to colistin in this microorganism and to investigate the relationship between acquisition of colistin resistance and virulence.

Materials and methods

Bacteria

An A. nosocomialis clinical isolate was identified using the Amplified Ribosomal DNA Restriction Analysis (ARDRA) method as described elsewhere. Colistin-resistant mutants were obtained by serial passages on plates with increasing concentrations of colistin sulphate salt (Sigma Aldrich). Mutant stability was corroborated by serial passages on non-selective medium (blood agar plates).

Antimicrobial susceptibility testing

The MICs of colistin for the WT and mutants of A. nosocomialis were determined using the broth microdilution and Etest assays, whereas those of other antibiotics were determined using the Etest method.

Caenorhabditis elegans model

The C. elegans infection assay was carried out with an A. nosocomialis colistin-susceptible strain with an MIC of 0.125 mg/L and the in vitro-selected resistant mutant with an MIC of 256 mg/L using the Fer-15 mutant line, which has a temperature-sensitive fertility defect. Fer-15 mutants were provided by the C. elegans Genetics Center. The model used has been described by Lavigne et al.

Statistical analysis

Kaplan–Meier survival curves were constructed to analyse the virulence data for each group of strains. Pairwise comparison between two different strains was carried out with a log rank test. A P value ≤0.05 obtained by GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) was considered statistically significant.

Genome sequencing

DNA from the strains sequenced was extracted using a DNA extraction kit (Promega). Genomic DNA from A. nosocomialis strains was fully sequenced by pyrosequencing using the genome sequencer Titanium (454 Life Sciences). A library of paired-end fragments was created following the manufacturer’s instructions (454 Life Sciences). This library was sequenced using the genome sequencer Titanium (454 Life Sciences). Reads originating from each strain were assembled into contigs using Newbler 2.53 (454 Life Sciences). The assembly was verified using the CLC Genomics software (CLC Bio). Predicted proteins were compared with a non-redundant GenBank database using BLASTP for functional annotation.

Limulus amoeocyte lysate assay

To determine the amount of endotoxin present in colistin-susceptible and colistin-resistant strains, the commercial kit QCL-1000 for limulus amoeocyte lysate assay (Lonza) was used following the manufacturer’s instructions.

Amplification of lipid A biosynthesis genes

The lipid A biosynthesis genes lpxA, lpxC and lpxD were amplified from A. nosocomialis genomic DNA by PCR using the following primers: lpxA forward, CCTATTGTATTAGTGCTC; lpxA reverse, CGTACATTCCAGCCTC; lpxC forward, GCGAGGGAAATAGGCTT; lpxC reverse, CGTATGAATTGGAGACTG; lpxD forward, GCCATGCACCTAAGTATA; and lpxD reverse, TCGCATGGAATTCAGGG.

Proteomic analysis

A description of the preparation of the outer membrane protein (OMP) extracts as well as the proteomic experimental procedures are available as Supplementary data at JAC Online.

Results and discussion

Selection of colistin-resistant A. nosocomialis mutants

An A. nosocomialis clinical isolate was subjected to serial passages on plates with increasing concentrations of colistin. For the A. nosocomialis WT strain selected the initial MIC was ≤0.5 mg/L. During mutant selection there was an inflection point (32 mg/L colistin in the plate for selection) where the strain showed a drastic change in MIC of colistin (from ≤0.5 to 32 mg/L). Strains isolated previously to this inflection point were found to tolerate up to 16 mg/L colistin. Other characteristics seen in the highly colistin-resistant mutants compared with the WT strain were that the mutants

Table 1. MIC of colistin and growth on MacConkey plates for selected A. nosocomialis strains together with their mutations in the lpxD gene and the amount of LPS

<table>
<thead>
<tr>
<th>Colistin in plate (mg/L)</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (mg/L)</th>
<th>Growth on MacConkey</th>
<th>Mutations in lpxD</th>
<th>LPS (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>1.74 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.25</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>0.5</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>6.24 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>≤0.5</td>
<td>yes</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>32</td>
<td>32</td>
<td>no</td>
<td>+</td>
<td>59.9</td>
</tr>
<tr>
<td>64</td>
<td>32</td>
<td>no</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>128</td>
<td>128</td>
<td>no</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>256</td>
<td>128</td>
<td>no</td>
<td>+</td>
<td>143</td>
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</tbody>
</table>


<sup>a</sup>Determined by the microdilution method.
had a change in colony morphology, with very small colonies being observed, and they did not grow on MacConkey agar (Table 1). At a given point, two different morphologies were observed, corresponding to colistin-susceptible and colistin-resistant strains. To our knowledge, this is the first description of a colistin-resistant mutant with a decreased ability to grow on MacConkey selective medium. This feature of the mutants could be associated with the fact that, on losing LPS, the strain does not tolerate the concentration of bile salts present in MacConkey agar.\textsuperscript{17}

**Antimicrobial resistance profile of the selected mutants**

Table 2 shows the MICs of different antimicrobial agents for the different mutant strains selected. Overall, the selected colistin-resistant mutants showed lower MICs of most of the antimicrobial agents tested. The MICs of cefoxitin and vancomycin fell from 24 to 1.5 mg/L and from 64 to 0.75 mg/L, respectively. For the carbapenems, imipenem and ertapenem, the decreases were from 0.5 to 0.064 mg/L and from 3 to 0.016 mg/L, respectively. The MIC of azithromycin for the colistin-resistant mutants decreased by four dilutions compared with the initial strain. In addition the MIC of tigecycline also decreased 3-fold. On comparing these values with the colistin-resistant A. baumannii strains, a similar, albeit less notable, behaviour was observed. López-Rojas et al.\textsuperscript{18} studied a patient with an infection by an A. baumannii strain susceptible to colistin, tigecycline, amikacin, gentamicin and tobramycin. After 34 days of different treatments, including colistin, gentamicin, sulfactam and cefepime, the strain became susceptible to all of the antibiotics apart from colistin.

**In vivo virulence using the C. elegans model**

One important factor of the new strains is their ability to cause virulence and the possible relationship between acquisition of resistance to colistin and changes in virulence. Therefore, we performed several experiments using a C. elegans model in order to investigate the virulence of the strains selected. Figure 1 shows a significant difference in virulence between the two strains, with the initial colistin-susceptible strain being more virulent compared with the colistin-resistant strain. The LT\textsubscript{50} (i.e. time required to kill 50\% of the nematodes) for the colistin-susceptible strain was 7 days compared with 9 days for the colistin-resistant strain. The LT\textsubscript{50} (i.e. time required to kill 50\% of the nematodes) for the colistin-susceptible strain was 7 days compared with 9 days for the colistin-resistant strain. The LT\textsubscript{50} (i.e. time required to kill 50\% of the nematodes) for the colistin-susceptible strain was 7 days compared with 9 days for the colistin-resistant strain. The LT\textsubscript{50} (i.e. time required to kill 50\% of the nematodes) for the colistin-susceptible strain was 7 days compared with 9 days for the colistin-resistant strain. The LT\textsubscript{50} (i.e. time required to kill 50\% of the nematodes) for the colistin-susceptible strain was 7 days compared with 9 days for the colistin-resistant strain.

**Sequence of the genome of the colistin-susceptible and colistin-resistant A. nosocomialis strains**

The genomes of the colistin-susceptible strain (WT) and the colistin-resistant mutant (256) of A. nosocomialis were sequenced. Comparison of the sequences of the genomes of the two strains showed several differences in the lpx\textsubscript{ACD} operon potentially related to colistin resistance that have been previously reported in A. baumannii, but not in the pmrABC operon. Several mutations (H196P, A199V, I206F, R211G, R220G, A222V, T226P, N236T, A251V, A254V, C256G, G257A, T262P, I264L, G265A, N267T, C268V and I269F and the 270 stop codon) were observed only in the colistin-resistant mutant (256) of A. nosocomialis. In addition to these mutations, a stop codon was observed in the pmrABC operon. Several mutations (H196P, A199V, I206F, R211G, R220G, A222V, T226P, N236T, A251V, A254V, C256G, G257A, T262P, I264L, G265A, N267T, C268V and I269F and the 270 stop codon) were observed only in the colistin-resistant mutant (256) of A. nosocomialis.
Table 3. Function and statistics of the proteins with a significant level of expression identified by DIGE on comparison of colistin-susceptible and colistin-resistant *A. nosocomialis* strains

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein ID</th>
<th>Protein</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANOVA P value</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>407441282</td>
<td>OmpA C-like</td>
<td>1.3</td>
<td>0.0181</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>490848590</td>
<td>hypothetical protein F984_02367 (NodT family RND efflux system)</td>
<td>3.4</td>
<td>0.00682</td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>479997515</td>
<td>hypothetical protein (tetratricopeptide repeat family protein)</td>
<td>6.3</td>
<td>0.0101</td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>593656836</td>
<td>putative carbapenem-associated resistance protein (CarO)</td>
<td>1.3</td>
<td>0.0697</td>
</tr>
<tr>
<td>4a</td>
<td>gi</td>
<td>593656836</td>
<td>putative carbapenem-associated resistance protein (CarO)</td>
<td>−1.5</td>
<td>0.0444</td>
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<tr>
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<td>gi</td>
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<td>putative carbapenem-associated resistance protein (CarO)</td>
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<td>0.00612</td>
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<tr>
<td>5</td>
<td>gi</td>
<td>466899273</td>
<td>OmpW-like protein</td>
<td>6.3</td>
<td>0.0101</td>
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<tr>
<td>6</td>
<td>gi</td>
<td>354459714</td>
<td>OmpA (isoform)</td>
<td>5.2</td>
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<td>7</td>
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<td>succinate dehydrogenase flavoprotein subunit</td>
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<td>0.00239</td>
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<tr>
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<td>gi</td>
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<td>porin (OprB)</td>
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<tr>
<td>9</td>
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<td>493628869</td>
<td>membrane protein (outer membrane β-barrel domain protein)</td>
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<td>0.0179</td>
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<tr>
<td>10</td>
<td>gi</td>
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<td>691132856</td>
<td>fatty-acyl-CoA synthase</td>
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<td>gi</td>
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<td>15</td>
<td>gi</td>
<td>588227534</td>
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<td>hypothetical protein</td>
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<td>690978220</td>
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<td>0.0213</td>
</tr>
<tr>
<td>19</td>
<td>gi</td>
<td>490848452</td>
<td>VacJ family lipoprotein</td>
<td>−28</td>
<td>0.00819</td>
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</table>

<sup>a</sup>Positive values mean an increased abundance and negative values mean a decreased abundance in the resistant strain.
the lpxD gene of the colistin-resistant strain. To determine when these mutations appeared we performed PCR and sequencing of the lpxD gene in all the different colistin-tolerant and -resistant A. nosocomialis mutants. The mutation generating the stop codon was also detected by PCR and sequencing in all of the highly colistin-resistant strains, but was not observed in the WT and colistin-tolerant strains. Two main different mechanisms of colistin resistance have been described in A. baumannii, a modification of LPS and a loss of LPS. We only observed mutations in the lpxD gene that stopped the production of LPS in A. nosocomialis. On studying the mechanism of resistance to colistin in A. baumannii, Moffatt et al.\textsuperscript{10} showed several mutations in all the genes in the lpxACD operon; our finding is the first description of a mutation generating a stop codon in the lpxD gene.

**LPS production of the WT and mutant A. nosocomialis strains**

It was hypothesized that the stop codon generated in the lpxD gene did not allow the bacteria to synthesize LPS. Therefore, the amount of endotoxin present in two colistin-susceptible and two colistin-resistant A. nosocomialis strains was determined, showing very low levels of endotoxin detection in the colistin-resistant mutants (Table 1). This is in agreement with what has been described in A. baumannii, in which all colistin-resistant strains with mutations in the lpxACD genes have decreased synthesis of LPS.\textsuperscript{10,21}

**OMP comparative proteomic analysis**

The combination of differential in-gel electrophoresis (DIGE) with the MALDI-TOF platform allowed the identification of proteomic changes in the OMP subproteome of A. nosocomialis in two isogenic strains: a colistin-susceptible (WT) strain and a colistin-resistant (256) strain (Figure S2). Using this technology we identified 19 spots with different abundance levels. Table 3 provides detailed information about the proteins identified as well as protein identification parameters.

The VacJ protein (spot 19) was among the proteins with modified abundance. The abundance of this protein was drastically reduced (28-fold change) in the colistin-resistant strain. This protein actively prevents phospholipid accumulation at the cell surface and probably maintains lipid asymmetry in the outer membrane by retrograde trafficking of phospholipids from the outer to the inner membrane.\textsuperscript{22} In Pseudomonas aeruginosa it has been demonstrated that this protein plays an important role in both antibiotic susceptibility and virulence.\textsuperscript{31}

The protein F984_02367 has been demonstrated to be involved in colistin resistance. This protein is annotated in A. nosocomialis as a hypothetical protein and it is an RND (resistance–nodulation–division) outer membrane efflux protein. It has been shown that this protein is required for antimicrobial resistance in Vibrio cholerae. Specifically, mutants of this protein in V. cholerae lead to increased susceptibility to the antibiotics erythromycin, polymyxin B and penicillin, but no changes in the antibiotic profile have been observed for kanamycin, nalidixic acid, ciprofloxacin, rifampicin, cefotaxime, carbencillin, tetracycline or chloramphenicol. The higher abundance of this protein in A. nosocomialis could also play a role in the increased resistance to colistin of this microorganism.

Apart from the above-mentioned proteins, interesting differences have been found in the abundance of the carbapenem-associated resistance protein (CarO). First of all we found three different isoforms (spots 4, 4a and 4b) of this protein differing only in molecular weight, but with the same isoelectric point, suggesting that the presence of some post-translational modifications causes the appearance of these isoforms. The presence of two isoforms with slight differences in molecular weight has been described previously. The isoform with the greatest molecular weight reportedly has a binding site for imipenem.\textsuperscript{24} We identified a third isoform. The abundance of two of the isoforms (spots 4a and 4b) decreased slightly in the comparative proteomic analysis, but one isoform (spot 4) showed a slight increase in the resistant strain. This protein showed a high specificity for imipenem, but not for meropenem. The decrease in the abundance of these two isoforms could explain the reduction in resistance to imipenem (Table 2).

Briefly, although a clear target responsible for the colistin-resistance acquisition has not been identified it seems evident that these two isogenic strains differ in membrane composition since key proteins involved in phospholipid accumulation at the cell surface (spot 19) as well as fatty acid biosynthesis (spot 13) showed modified levels of abundance in the subproteome analysed. These results agree with the previously published results by Henry et al.\textsuperscript{25} These authors performed a comparative transcriptional analysis of a colistin-susceptible A. baumannii against a colistin-resistant isogenic strain of A. baumannii and found differential expression of the genes involved in membrane biogenesis, lipoprotein transport and exopolysaccharide production. Although we only performed comparative proteomic analysis with the outer membrane subproteome, as in the study by Henry et al.,\textsuperscript{25} our proteomic results suggest that the membrane composition of the resistant strain of A. nosocomialis is modified to compensate for the loss of LPS.

**Conclusions**

In summary, in this study we have shown that the mechanism of resistance to colistin in A. nosocomialis is mainly associated with the loss of LPS due to mutations and inactivation of the lpxD gene, although changes in the expression of some proteins cannot be ruled out. In addition, the acquisition of colistin resistance is related to a decrease in virulence.

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**Transparency declarations**

None to declare.

**Supplementary data**

Supplementary Materials and methods and Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
References


