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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 LT_{50} (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).[1] Resistance to carbapenems has been associated with the acquisition of carbapenemases,[1] with some of these carbapenemases, such as OXA-23,[2] OXA-58[3] 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%.[11–14] 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.[15] 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 obtained 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 was provided by the *Caenorhabditis* Genetics Center. The model used has been described by Lavigne *et al*.[16]

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 amoebocyte lysate assay

To determine the amount of endotoxin present in colistin-susceptible and colistin-resistant strains, the commercial kit QCL-1000 for limulus amoebocyte 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, CCTTATTGTATTATTGGTCC; lpxA reverse, CGTACAATTCCACGCTC; lpxC forward, GCGAGTGGAATAGGTCTT; lpxC reverse, CGTATGGAATTGGACAGTC; lpxD forward, GCCTATGACGCTAAGTATGA; and lpxD reverse, TCTGCATTGGTAATTCAGGG.

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 the 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

Colistin in plate[a] (mg/L)	MIC[b] (mg/L)	Growth on MacConkey	Mutations in *lpxD*	LPS (EU/mL)
0	≤0.5	yes	−	1.74×10^5
0.25	≤0.5	yes	−	ND
0.5	≤0.5	yes	−	ND
1	≤0.5	yes	−	ND
2	≤0.5	yes	−	6.24×10^4
4	≤0.5	yes	−	ND
8	≤0.5	yes	−	ND
16	≤0.5	yes	−	ND
32	32	no	+	59.9
64	32	no	+	ND
128	128	no	+	ND
256	128	no	+	143

EU, endotoxin units; ND, not determined; +, mutations were found in amino acid codons H196P, A199V, I206F, R211G, R220G, A222V, T226P, N236T, A251V, A254V, C256G, G257A, T262P, I264L, G265A, N267T, C268V and I269F and the 270 stop codon.
[a]Concentration used to select resistant mutants.
[b]Determined by the microdilution method.

Table 2. MICs of several antibacterial agents for the WT and colistin-tolerant and colistin-resistant *A. nosocomialis* mutants

	MIC[a] (mg/L)									
Colistin in plate (mg/L)	FOX	CAZ	ATM	IPM	ETP	VAN	CIP	TOB	TGC	CST
0	24	2	0.38	0.5	3	64	0.125	0.38	0.125	≤1
2	24	2	0.38	0.5	3	64	0.125	0.38	0.125	≤1
8	24	2	0.38	0.5	3	64	0.125	0.38	0.125	≤1
16	24	2	0.38	0.5	3	64	0.125	0.38	0.125	≤1
32	1.5	0.75	0.047	0.064	0.016	0.75	0.064	0.19	0.094	128
256	1.5	0.75	0.047	0.064	0.016	0.75	0.064	0.19	0.047	512

FOX, cefoxitin; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; ETP, ertapenem; VAN, vancomycin; CIP, ciprofloxacin; TOB, tobramycin; TGC, tigecycline; CST, colistin.
[a]Determined by Etest.

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.[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.*[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, sulbactam 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_{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

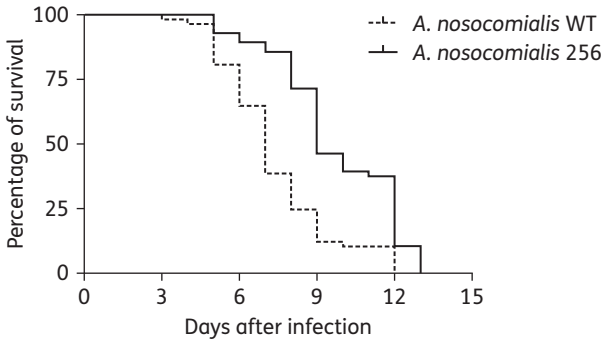

Figure 1. Percentage of survival after infection with two different strains (colistin-susceptible and colistin-resistant *A. nosocomialis*) using the *C. elegans* killing assay. *A. nosocomialis* WT versus *A. nosocomialis* 256: $P<0.0001$.

($P<0.0001$). On the other hand, the LT_{50} of the control strain of *E. coli* was 11 days (data not shown). The same decrease in the virulence associated with the acquisition of resistance to colistin was observed in *A. baumannii* in both *in vitro* and *in vivo* studies;[19] however, fitness and virulence differed depending on the mechanism of colistin resistance.[20] However, in our study the fitness of the different mutants was not statistically different compared with the WT strain (Figure S1).

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 *lpxACD* 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 *lpxD* gene on comparing the genome sequences of colistin-susceptible and -resistant *A. nosocomialis* isogenic strains. It is important to highlight that one of these mutations generated a stop codon in

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

Spot number	Protein ID	Protein	Fold change[a]	ANOVA *P* value	Function
1	gi|407441282	OmpA_C-like	1.3	0.0181	peptidoglycan binding domains similar to the C-terminal domain of OMP OmpA
2	gi|490848590	hypothetical protein F984_02367 (NodT family RND efflux system)	3.4	0.00682	these proteins work with an inner membrane ABC transporter ATPase and an adapter called a membrane fusion protein; most members of this family are likely to export primarily small molecules rather than proteins, but are related to the type I protein secretion OMPs TolC and PrtF
3	gi|479997515	hypothetical protein (tetratricopeptide repeat family protein)	6.3	0.0101	protein–protein interaction and protein complex formation
4	gi|593656836	putative carbapenem-associated resistance protein (CarO)	1.3	0.0697	carbapenem-associated resistance protein
4a	gi|593656836	putative carbapenem-associated resistance protein (CarO)	−1.5	0.0444	carbapenem-associated resistance protein
4b	gi|593656836	putative carbapenem-associated resistance protein (CarO)	−1.7	0.00612	carbapenem-associated resistance protein
5	gi|446899273	OmpW-like protein	6.3	0.0101	homologue of OmpW with unknown function
6	gi|354459714	OmpA (isoform)	5.2	0.00181	porin function, adhesion and invasion
7	gi|487978330	succinate dehydrogenase flavoprotein subunit	2.3	0.00239	member of citric acid cycle and the respiratory chain
8	gi|487978520	porin (OprB)	−2.1	0.00233	carbohydrate-selective porin
9	gi|493628869	membrane protein (outer membrane β-barrel domain protein)	2	0.0179	unknown protein function
10	gi|487981035	signal peptide protein	1.7	0.0372	putative MetA pathway of phenol degradation; some proteins in this family are classified by the Transporter Classification Database (TCDB; www.tcdb.org) as belonging to the putative β-barrel porin/α amylase or MetA pathway of the phenol degradation superfamily
11	gi|587819016	putative porin	1.4	0.0713	putative 34 kDa OMP
12	gi|691122992	outer membrane receptor protein FepA	−3.7	0.00167	active transport of iron from the extracellular space into the periplasm of Gram-negative bacteria; FepA has also been shown to transport vitamin B12, and colicins B and D as well
13	gi|691132856	fatty-acyl-CoA synthase	−1.6	0.00584	fatty acid biosynthesis
14	gi|554761160	TonB-dependent siderophore receptor	−2	0.000662	involved in active transport of iron chelators (siderophores), oligosaccharides and polypeptides
15	gi|588227534	putative selenocysteine synthase	−2.9	0.000816	synthesis of selenocysteine
16	gi|491026500	hypothetical protein	2.1	0.00367	unknown protein function; this protein contains two META domains; proteins containing this domain are secreted and implicated in motility in bacteria
17	gi|491220730	copper resistance protein CopB	1.8	0.00428	the Cop proteins apparently mediate sequestration of copper outside of the cytoplasm as a copper-resistance mechanism
18	gi|690978220	outer membrane porin, OprD	−1.5	0.0213	specific porin, which facilitates uptake of basic amino acids and imipenem, a carbapenem antibiotic
19	gi|490848452	VacJ family lipoprotein	−28	0.00819	actively prevents phospholipid accumulation at the cell surface

[a]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 *lpx*D 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*.[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.[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.[22] In *Pseudomonas aeruginosa* it has been demonstrated that this protein plays an important role in both antibiotic susceptibility and virulence.[23]

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, carbenicillin, 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.[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*.[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*.,[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/).

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