which should be cited to refer to this work.

Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*

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Gene modifications in the PmrAB and PhoPQ two-component regulatory systems, as well as inactivation of the *mgrB* gene, are known to be causes of colistin resistance in *Klebsiella pneumoniae.* The objective of this study was to characterise the mechanism involved in colistin resistance in a *Klebsiella oxytoca* isolate. A *K. oxytoca* clinical isolate showing resistance to colistin was recovered in Cali, Colombia. The *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* genes were amplified and sequenced. Wild-type *mgrB* genes from *K. pneumoniae* and *K. oxytoca* were cloned, and corresponding recombinant plasmids were used for complementation assays. By analysing the *mgrB* gene of the *K. oxytoca* isolate and its flanking sequences, an insertion sequence (IS) of 1196 bp was identified in its promoter region. The insertion was located between nucleotides −39 and −38 when referring to the start codon of the *mgrB* gene, thus negatively interfering with expression of the *mgrB* gene by modifying its promoter structure. This IS was very similar to IS*Kpn26* (99% nucleotide identity) belonging to the IS*5* family. Complementation assays with *mgrB* genes from wild-type *K. pneumoniae* or *K. oxytoca* restored full susceptibility to colistin. In conclusion, here we identified the mechanism involved in colistin resistance in a *K. oxytoca* isolate. Modulation of *mgrB* gene expression was the key factor for this acquired resistance to colistin.

1. Introduction

Klebsiella oxytoca and *Klebsiella pneumoniae* are frequent sources of nosocomial infections and the sources of nosocomial outbreaks [1,2]. The occurrence of multidrug resistance is increasingly observed among the Gram-negative pathogens *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae. Consequently, use of colistin, a polymyxin-type antibiotic, is being reconsidered in particular for treating critically ill patients infected with these pathogens [3–5]. Unfortunately, the increasing usage of colistin is being associated with the emergence of colistin resistance, in particular in *K. pneumoniae* [6–11].

Alterations in the two-component regulatory systems PmrAB and PhoPQ [8,9,12] are known to be involved in polymyxin resistance in *K. pneumoniae*. Inactivation of the *mgrB* gene, encoding a negative feedback regulator of the PhoPQ two-component system, is also a common mechanism involved in colistin resistance in *K. pneumoniae* [10,13]. The reported alterations in the *mgrB* gene include insertion of several insertions sequences (IS*5*-like, IS*1F*-like, IS*Kpn13*, IS*Kpn14*) at different locations in the coding sequence of *mgrB* and in its promoter region. Non-silent point mutations, premature stop codons, small intragenic deletions and large deletion of the *mgrB* locus are also known. These alterations result in either reduced or even lack of expression of the *mgrB* gene leading to upregulation of the *phoPQ* and *pmrHFIJKLM* operons that confer resistance to colistin.

Lippa and Goulian have identified MgrB homologues in the genome sequences of various enterobacterial species [14]. Therefore, alterations in *mgrB* gene expression might possibly be involved in acquisition of colistin resistance in different *Klebsiella* species.

The aim of this study was to identify the mechanism responsible for resistance to colistin in a *K. oxytoca* clinical isolate.

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2. Materials and methods

2.1. Bacterial strain

The colistin-resistant *K. oxytoca* clinical isolate was identified using an API20E system and the matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) technique (AB bioMérieux, La Balme-les-Grottes, France). A colistin-susceptible *K. oxytoca* clinical isolate was used as a wild-type control.

2.2. Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using Etest strips (AB bioMérieux) on Mueller–Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France) with a 0.5 McFarland inoculum. Minimum inhibitory concentrations (MICs) were interpreted as indicated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0, 2014; http://www.eucast.org). Isolates with colistin MICs of $\leq$2 μg/mL were categorised as susceptible whereas those with MICs of >2 μg/mL were resistant.

2.3. PCR amplification and sequencing

Whole-cell DNA was extracted using a QIAquick Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. The entire *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* genes were amplified with specific oligonucleotides primers (Table 1) designed with sequences of *K. oxytoca* KCTC 1686 and E718 genomes available on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov.gate2.inist.fr/). Amplified DNA fragments were purified with a QIAquick PCR Purification Kit (QIAGEN). Both strands of the amplification products obtained were sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analysed at the NCBI website (http://www.ncbi.nlm.nih.gov) by the Basic Local Alignment Search Tool (BLAST) programme. The insertion sequence (IS) was analysed using the IS finder website (http://www-is.biotoul.fr).

2.4. Complementation assays

The wild-type *mgrB* gene from a colistin-susceptible *K. oxytoca* strain and a *K. pneumoniae* strain, as well as the non-coding *mdh* sequence [15], were amplified by PCR using 2X Phusion® HF Master Mix (Finnzymes; Life Technologies, Illkirch, France) and the primers listed in Table 1. The amplified fragments were cloned into the plasmid pCR®-Blunt II-TOPO® (Invitrogen, Illkirch, France) and the resulting plasmids pTOPO-*mgrBKox*, pTOPO-*mgrBKp* and pTOPO-*mdh* (encoding resistance to Zeocin™) were respectively transformed into an electrocompetent colistin-resistant *K. oxytoca* isolate by electroporation. Electrotransformants were selected by overnight incubation at 37 °C on Mueller–Hinton agar supplemented with 100 μg/mL Zeocin™. MICs of colistin for the *K. oxytoca* transformants were determined by Etest (bioMérieux).

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Gene	Ref.
Kox pmrC ext F	GCC GAT ATC GCA GGG GTT AA	*pmrC*	This study
Kox pmrC ext R	TAA CAG GAG CGC ATC GTC TT	*pmrC*	
Kox pmrA ext F	GCA AGC GTA TTC GCA GGA TA	*pmrA*	
Kox pmrA ext R	CTG ATG AGC TGA CAA ACG GC	*pmrA*	
Kox pmrB ext F	CAA CGA CAT TTA CAG CTG GG	*pmrB*	
Kox pmrB ext R	CTT TAC TGA GGA TAG CGC CA	*pmrB*	
Kox phoP ext F	CCA AGA GAC CGA GGT ACA AA	*phoP*	
Kox phoP ext R	GAG TGA CAA CAC CAG CAC TA	*phoP*	
Kox phoQ ext F	CCA TAC CAT CGA TGT GCT GA	*phoQ*	
Kox phoQ ext R	GCA GGT GTC TGT TAG GGA TT	*phoQ*	
Kox mgrB ext F	CGC GGT TTA AGA AGG TCA TG	*mgrB*	
Kox mgrB ext R	AGG CGT TTA TTC TAC CAC CC	*mgrB*	
mdh F	CCC AAC TCG CTT CAG GTT CAG	*mdh*	[15]
mdh R	CCG TTT TTC CCC AGC AGC AG	*mdh*	

3. Results and discussion

3.1. Strain and patient features

The colistin-resistant *K. oxytoca* isolate C24 was recovered in February 2008 from a soft-tissue secretion from a 66-year-old male patient hospitalised for an intra-abdominal infection in Cali, Colombia. The isolate was resistant to broad-spectrum cephalosporins (MICs of ceftazidime and cefotaxime = 32 μg/mL), amikacin (MIC = 16 μg/mL) and colistin (MIC = 24 μg/mL) and was of intermediate susceptibility to ciprofloxacin (MIC = 1 μg/mL). The isolate was susceptible to carbapenems (MIC < 0.5 μg/mL), gentamicin (MIC = 0.5 μg/mL) and tigecycline (MIC = 0.5 μg/mL). It harboured a $bla_{\text{CTX-M-15}}$ gene but did not produce any carbapenemase according to the Carba NP test. To our knowledge, the patient had never been treated with any polymyxin.

3.2. Modification of mgrB gene expression through insertion of an insertion sequence element

Sequence analysis of the *pmrA*, *pmrB*, *phoP* and *phoQ* genes known to be involved in lipopolysaccharide (LPS) synthesis showed 100% identity with the genes identified from colistin-susceptible wild-type *K. oxytoca* isolates (data not shown). Sequence analysis of the *mgrB* gene as well as upstream-located sequences revealed that an IS of 1196 bp had targeted the upstream vicinity of the *mgrB* gene between nucleotides −38 and −39 when referring to the start codon of *mgrB*. This IS was similar to IS*Kpn26* (differing by only 5 bp) and belonged to the IS*5* family. It was bracketed by a 4-bp duplication (TTAT) being the likely signature of a transposition process. The origin of IS*Kpn26* is *K. pneumoniae* (https://www-is.biotoul.fr/index.html?is_special_name=ISKpn26) and it has also been identified in *K. oxytoca* KONIH1 (GenBank accession no. CP008788.1). In silico analysis of the sequence located upstream of a wild-type *mgrB* gene revealed a putative promoter sequence made of −35 [TTGAAA] and −10 [TAAACT] boxes separated by 15 bp (Fig. 1). Insertion of IS*Kpn26* in isolate C24 led to displacement of this promoter with respect to the *mgrB* gene position, the corresponding −35 and −10 sequences being consequently very distant from it (Fig. 1). This suggests a decrease or even lack of expression of the *mgrB* gene, leading to low or absence of production of the corresponding protein and consequently to acquired resistance to colistin.

3.3. Complementation experiments

To confirm whether higher expression of *mgrB* might restore susceptibility to colistin, complementation of isolate C24 with a plasmid overexpressing *mgrB* was performed. Transformation assays were either performed with plasmids encoding the MgrB protein of *K. oxytoca* or *K. pneumoniae*, respectively. The *mgrB* gene was provided in trans together with its original promoter in order to ensure it would be expressed at a wild-type level. Following complementation, isolate C24 recovered full susceptibility to colistin with the wild-type MgrB of *K. oxytoca* (MIC = 0.047 μg/mL). Interestingly, the same MIC was obtained after complementation with the MgrB of *K. pneumoniae* (MIC = 0.047 μg/mL). As expected,

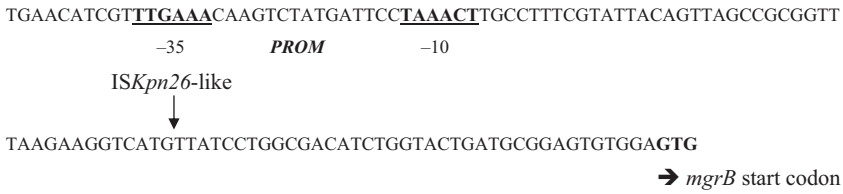

Fig. 1. Sequences located at the 5′-extremity of the *mgrB* gene. The putative promoter (*PROM*) is indicated, with its respective −35 and −10 boxes underlined. The arrow indicates the target site for insertion of the insertion sequence IS*Kpn26*-like. The *mgrB* start codon is in bold.

transformation with plasmid pTOPO-*mdh* used as a negative control did not restore susceptibility to colistin (MIC = 24 μg/mL).

3.4. Comparison of mgrB gene sequences of K. oxytoca and K. pneumonia

Alignment of the *mgrB* sequences from wild-type *K. pneumoniae* MGH78578 and *K. oxytoca* KCTC1686 strains showed that the sequences were almost identical [only a single amino acid substitution (Cys to Tyr at amino acid position 28)]. This result explains why complementation with the *mgrB* gene of *K. pneumoniae* restored full susceptibility to colistin in the *K. oxytoca* isolate. Furthermore, it may suggest that all previously reported alterations of the *mgrB* gene [10,13] that have been shown to be the sources of acquired resistance to colistin in *K. pneumoniae* (such as insertions of IS elements at different locations in the coding sequence of *mgrB*, non-silent point mutations, premature stop codons, intragenic deletions, etc.) might also be a source of colistin resistance in *K. oxytoca*.

4. Conclusion

This study reports on the molecular identification of colistin resistance in *K. oxytoca.* This resistance mechanism is associated with modification of the biosynthesis pathway of LPS. It is similar to that reported in *K. pneumoniae*, underlining that common mechanisms of colistin resistance could be identified in different enterobacterial species.

Nucleotide sequence accession number

The nucleotide sequences of the mutated *mgrB* genes identified in this study have been deposited at DDBJ/EMBL/GenBank under accession no. KP748172.

Funding

This work was funded by the University of Fribourg (Fribourg, Switzerland) and by a grant from the European Community [MAGIC-BULLET, FP7/HEALTH-F3-2001-278232].

Competing interests

None declared.

Ethical approval

Not required.

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