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## COLISTIN RESISTANCE ASSOCIATED WITH OUTER MEMBRANE PROTEIN CHANGE IN *KLEBSIELLA PNEUMONIAE* AND *ENTEROBACTER ASBURIAE*

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In this study, outer membrane proteins (OMPs) of colistin-resistant *Klebsiella pneumoniae* and *Enterobacter asburiae* were analyzed. One colistin-susceptible and three colistin-resistant *K. pneumoniae* sequence type 258 strains as well as one colistin-susceptible *E. asburiae* and its colistin-heteroresistant counterpart strain were involved in the study. OMP analysis of each strain was performed by microchip method. Matrix-assisted laser desorption ionization time of flight/mass spectrometry (MALDI-TOF/MS) investigation was carried out after separation of OMPs by two-dimensional gel electrophoresis and in-gel digestion. The MALDI-TOF/MS analysis of OMPs in the colistin-susceptible *K. pneumoniae* found 16 kDa proteins belonging to the LysM domain/BON superfamily, as well as DNA starvation proteins, whereas OmpX and OmpW were detected in the colistin-resistant counterpart strains. OmpC and OmpW were detected in the colistin-susceptible *E. asburiae*, whereas OmpA and OmpX were identified in the colistin-resistant counterpart. This study demonstrated that OMP differences were between colistin-susceptible and -resistant counterpart strains. The altered Gram-negative cell wall may contribute to acquired colistin resistance in Enterobacteriaceae.

**Keywords:** MALDI-TOF/MS, outer membrane protein, colistin resistance

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## Introduction

*Klebsiella pneumoniae* and *Enterobacter* spp. are frequently identified nosocomial Gram-negative pathogens and both are found to be resistant to multiple classes of antibiotics, including extended-spectrum cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones [1–3].

The emergence of colistin resistance in *K. pneumoniae* was reported in several countries, and the sequence type (ST) 258 clone was most frequently identified, although strains from numerous clones (ST14, ST15, ST70, ST101, ST273, ST512, and ST1271) were also detected as colistin-resistant ones. Colistin resistance also appeared in *Enterobacter* spp.; however, it has been detected less frequently [4–9].

The main mechanism of resistance to colistin in Gram-negative bacteria is explained by the modification of lipopolysaccharide (LPS), the target molecule of polymyxins. The addition of phosphoethanolamine and 4-deoxyaminoarabinose to the target molecule reduces its affinity to positively charged agents like polymyxins. The *pmrCAB* operon encodes the PmrC phosphoethanolamine transferase, the PmrA response regulator (also named as BasR), and the PmrB sensor kinase (also named as BasS), whereas the *arn* operon encodes the enzymes and transports proteins responsible for 4-deoxyaminoarabinose substitution [10–14].

The outer membrane bound two-component regulatory systems PmrA/PmrB and PhoP/PhoQ were identified as major contributing factors in resistance to polymyxins. The insertional inactivation of the *mgrB* gene encoding another outer membrane protein (OMP) regulating PhoQ/PhoP has also recently been associated with colistin resistance [15–17].

In Hungary, the first colistin-resistant *K. pneumoniae* strains were detected between 2008 and 2009 during an outbreak of a *K. pneumoniae* carbapenemase-2 (KPC-2) producing ST258 clone. Later, increasing number of acquired colistin-resistant Enterobacteriaceae clinical isolates was observed. The aim of this study was to analyze one colistin-susceptible and three colistin-resistant clinical *K. pneumoniae* ST258 strains originating from the first Hungarian outbreak. This study also involved one colistin-susceptible *Enterobacter asburiae* and its colistin-heteroresistant counterpart strain. The specific aim of this study was the analysis of OMPs [18].

## Materials and Methods

### *Bacterial strains*

Three colistin-resistant and one colistin-susceptible KPC-2 producing *K. pneumoniae* ST258 strains were included in this study. All investigated strains

were identified in 2008–2009 during an outbreak in Hungary. One *E. asburiae* strain identified from clinical urine sample and its colistin-heteroresistant counterpart were also analyzed [18].

### *Isolation of OMPs*

The strains were inoculated into 500 ml Mueller–Hinton Broth (Oxoid Ltd., Basingstoke, UK) and were incubated in a shaker at 37 °C overnight. The cultures were centrifugalized (6,000g, 20 min, 4 °C), then the sediments were resuspended in physiological saline solution. The suspensions were again centrifugalized and this washing step was repeated once more. The sediments were subsequently taken into 15 ml 20 mM Tris-HCl (pH 7.5) solution, then the bacterial cells in ice bath were disrupted with 500W ultrasound (MSE Soniprep 150 Ultrasonic Disintegrator, MSE Ltd., London, UK) twice for 2 min. Hereupon, the samples were again centrifugalized (6,000g, 20 min, 4 °C), and after that, the supernatants were poured off for ultracentrifugation (100,000g, 60 min, 4 °C). Thereafter, the sediments were resuspended in 5 ml 0.5% *N*-laurylsarcosine (Sigma-Aldrich, Budapest, Hungary) solution and incubated at room temperature for 30 min, and then they were again ultracentrifugalized with same parameters. Finally, the sarcosine-insoluble OMPs were located in the sediment [19].

### *OMP analysis by microchip (Agilent 2100 Bioanalyzer)*

Electrophoresis in microchips was performed in the commercially available Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode laser for fluorescence detection with 630 and 650 nm as excitation and emission wavelengths, respectively. OMP samples were diluted 10 times with standard labeling buffer. For the fluorescent labeling, 0.5 µL of fluorescent dye/dimethyl sulfoxide solution was added to 5 µL of diluted sample and incubated for 10 min at room temperature. The excess dye (i.e., the unbound dye) in the solutions of labeled OMPs was quenched by adding 0.5 µL of ethanolamine following the reaction time. The labeled samples were diluted five times by adding 24 µL of distilled water, then incubated at 100 °C for 5 min. The samples were centrifugalized and the supernatant was used for the electrophoretic analysis, where the microchip channels were hydrodynamically filled (with pressure) with the gel matrix, the sample wells were loaded with 6 µL of samples, and the respective wells were loaded with the destaining solution. The injection was made with 1,000 V for 80 s and the separation was continued at 1,000 V for 60 s. The sample components migrated toward the anode. The temperature was maintained at 30 °C.

### *Two-dimensional gel electrophoresis (2DE) of OMPs*

OMPs of *K. pneumoniae* strains were separated by 2DE. A portion of 100 µg of the total protein content of the OMPs were supplemented with 2DE sample buffer 8 M urea (Bio-Rad, Budapest, Hungary), 2% CHAPS (Bio-Rad), 50 mM dithiothreitol (DTT) (Bio-Rad), 0.2% Biolyte 3/10 ampholytes (Bio-Rad), and a trace of bromophenol blue (Bio-Rad) to a total volume of 125 µl, then the immobilized pH gradient strips of length 7 cm, pH 3–10, (Bio-Rad) were incubated for rehydration overnight. Isoelectric focusing (IEF) of the OMPs was performed on an IEF cell (Bio-Rad) using the following program: 250 V, 2 h, linear, 500 V, 2 h, linear, 4,000 V, 10,000 Vh, rapid. After the IEF, the strips were equilibrated twice for 10 min in equilibration buffer containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, a trace of bromophenol blue, and 2% DTT (Bio-Rad). During the second equilibration step, 2.5% IAA was used instead of DTT. After the equilibration, the strips were applied to the second dimension [12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 8 × 6 cm (Bio-Rad)]. The separation of the proteins according to their masses was performed at 80 V for 20 min and 120 V until the end of the run. After the SDS-PAGE, the gels were stained with Coomassie blue R-250. Protein marker (ladder, Bio-Rad Precision Plus Protein™ Kaleidoscope™, Bio-Rad) was used as a molecular standard. The gels were scanned on Pharos FX laser scanner (Bio-Rad). For the identification of the OMPs and the mass spectrometric analysis, the spots of interest were excised from the gels.

### *In-gel digestion*

Protein bands were excised from gels and were cut to small pieces and digested using modified version of the protocol developed by Shevchenko et al. [20]. Coomassie blue and SDS were removed with 100 mM ammonium bicarbonate (Bio-Rad), then the gel slabs were dehydrated with acetonitrile (ACN). Disulfide bridges were reduced with 10 mM DTT (Bio-Rad), then the free sulfhydryl (SH) groups were alkylated with 55 mM iodoacetamide solution (Bio-Rad). The modified proteins were in-gel digested with side-chain-protected trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate overnight at 37 °C. The digested peptides were extracted from the gel with 5% formic acid solution (Sigma-Aldrich) in a 2:1 mixture of ACN (Sigma-Aldrich) and water. The extracted peptides were evaporated to dryness and redissolved in 5 µL of 0.1% trifluoroacetic acid (TFA) in water before the mass spectrometric measurement [20, 21].

### *Matrix-assisted laser desorption ionization time of flight/mass spectrometry (MALDI-TOF/MS)*

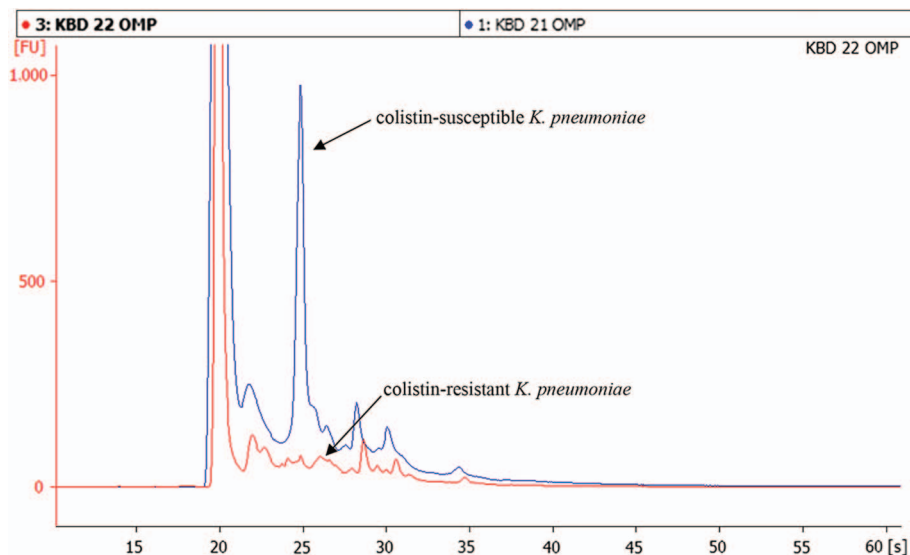
Mass analysis was performed on an Autoflex II MALDI-TOF/MS instrument (Bruker Daltonics, Bremen, Germany). During MS analysis of the digested proteins, 8 mg of  $\alpha$ -cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) was dissolved in 1 mL of 50% ACN and 0.1% TFA (Scharlau Chemie, Barcelona, Spain) in water. In each case, 1  $\mu$ L of the matrix was deposited on a stainless steel target together with 1  $\mu$ L of the sample with dried droplet method.

All mass spectra were monitored in positive mode with pulsed ionization [ $\lambda = 337$  nm; nitrogen laser (MNL 106 PD)] with a maximum pulse rate of 50 Hz. Peptides of the digests were measured in positive reflectron mode using a delayed extraction of 120 ns. The accelerating voltage was set to +19 kV and the reflectron voltage was set to +20 kV. The spectra of peptides were the sum of 1,000 shots; external calibration has been implemented. Data processing was executed with flexAnalysis software packages (version: 3.1; Bruker Daltonics, Bremen, Germany). Sequence Editor software (Bruker Daltonics) was used for the analysis with the following criteria: (i) all cysteines were supposed to be treated with iodoacetamide, (ii) monoisotopic masses were allowed, and (iii) the maximum number of missed cleavage sites was two [22, 23]. Data analysis was performed and protein identification was achieved using the MASCOT algorithm (<http://www.matrixscience.com>) and the Swiss-Prot entries database (Swiss Institute of Bioinformatics, Geneva, Switzerland), where (i) carbamidomethyl cysteine as fixed modification and (ii) oxidation of methionine were allowed as variable modifications. Mass accuracy was considered within 150 ppm for MS and 0.8 Da for MS/MS. Only proteins with at least two matching sequences were considered [20, 24].

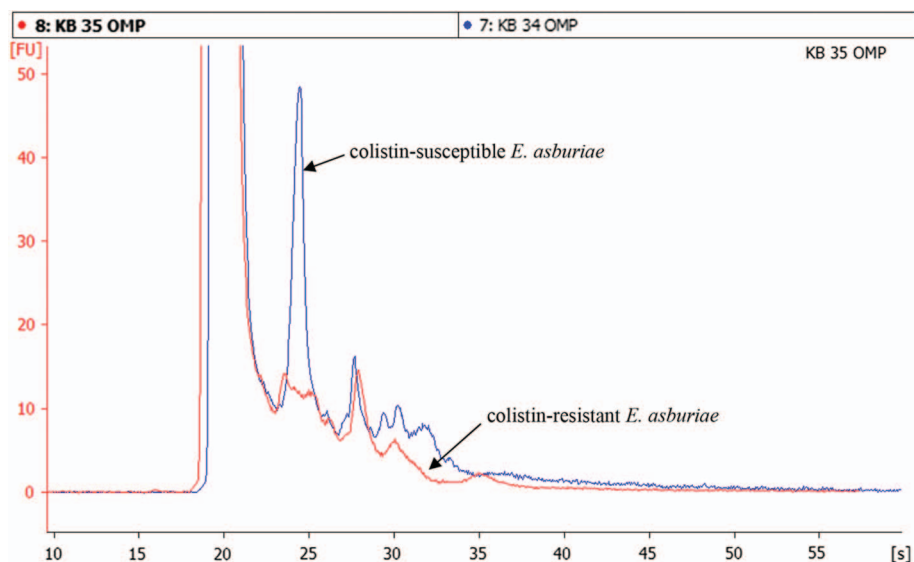
## **Results and Discussion**

Our investigations regarding the colistin resistance mechanisms found OMP change in the colistin-resistant strains compared with the colistin-susceptible ones. The Agilent 2100 Bioanalyzer showed a distinct lack of protein fraction in the colistin-resistant *K. pneumoniae* and *E. asburiae* isolates during the runs of OMPs, whereas the same fraction was present in the colistin-susceptible ones (Figures 1 and 2).

Various OMPs were detected by MALDI-TOF/MS after 2DE separation of OMPs (Table I). In the colistin-susceptible *K. pneumoniae* strain, 15–16 kDa



**Figure 1.** OMP analysis of colistin-susceptible and colistin-resistant *K. pneumoniae* strains (colistin-susceptible strain: blue line; colistin-resistant strain: red line)



**Figure 2.** OMP analysis of colistin-susceptible *E. asburiae* strain and its heteroresistant counterpart (colistin-susceptible strain: blue line; colistin-resistant strain: red line)

**Table I.** OMPs detected in colistin-susceptible and colistin-resistant *K. pneumoniae* and *E. asburiae*

Strain	Protein	Protein ID	Molecular weight (Da)
col S <i>Kpn</i> 11	LysM BON superfamily	gi 152970277	15,899
	DNA starvation protein	gi 152969398	18,697
col R <i>Kpn</i> 12	OmpW	gi 152969802	24,321
col R <i>Kpn</i> 97	OmpW, colicin S4 rec; putative transport protein	A6T7W0	24,321
	OmpX	S5YR85	14,866
col R <i>Kpn</i> 105	OmpX	F0JWH6	18,680
	OmpW	G0GLV0	22,969
	OmpC	K4YC30	40,459
col S <i>Easb</i>	OmpW	G0GLV0	22,969
	flagellin	V3HMK5	28,529
col R <i>Easb</i>	OmpA	V3PJ21	37,748
	OmpX	V3E259	18,654

Note: col S *Kpn*: colistin-susceptible *K. pneumoniae*; col R *Kpn*: colistin-resistant *K. pneumoniae*; col S *Easb*: colistin-susceptible *E. asburiae*; col R *Easb*: colistin-resistant *E. asburiae*.

proteins of the LysM domain /BON superfamily and DNA starvation/stationary phase protection proteins were detected, whereas in the colistin-resistant counterpart strain, these proteins were absent, but OmpX (19 kDa) and OmpW (24 kDa) were present. In the colistin-susceptible *E. asburiae*, OmpC (40 kDa) and OmpW (23 kDa) were identified, whereas in the colistin-heteroresistant counterpart strain, OmpA (38 kDa) and OmpX (19 kDa) were detected.

DNA starvation proteins (Dps) were found in several bacteria, their main function being the protection of bacteria during the stationary phase of cell division. They bind to the bacterial chromosome in a non-specific way creating a stable, condensed DNA starvation/stationary phase protection protein (Dps-DNA) complex which is protected against harmful effects. Furthermore, they bind intracellular  $Fe^{2+}$  ions and oxidize them into  $Fe^{3+}$  ions, then store them indirectly decreasing the amount of intracellular reactive oxygen species [25, 26].

LysM and BON domains are the conserved sections found in many bacterial proteins. These are mainly structural proteins and enzymes responsible for maintaining cell membrane integrity, and they are present in the greatest amount during the stationary cell division phase. The LysM domain specifically binds to the *N*-acetylglucosamine molecules of peptidoglycan. As colistin resistance is based on molecular changes in the cell wall, the alterations in the expression of these structural proteins may contribute to its development [22, 27].

OmpC and its homologues (Omp36 and OmpK36) are porin-type transport proteins found in the outer membrane of *Enterobacteriaceae*. They are responsible for transporting several types of molecules into the cells, including antibiotics

(e.g., cephalosporins, carbapenems, and fluoroquinolones). Their loss or decreased expression leads to antibiotic resistance and diminished susceptibility to serum antimicrobial activity in *Escherichia coli* and *Enterobacter* spp. [23, 28].

OmpA is a multifunctional membrane protein: in addition to maintaining integrity of the outer membrane, it is responsible for serum resistance in *E. coli* and antimicrobial peptide resistance in *K. pneumoniae* [29, 30].

OmpX is a protein structurally similar to OmpA. Its overproduction was observed in multiresistant *Enterobacter aerogenes* strains with the simultaneous underproduction of OmpF and Omp36 porins, as well as structural changes of LPS. Upregulation of *ompX* and downregulation of *omp36* together cause the decrease of outer membrane permeability [31–33].

The reports of OmpA and OmpX contributing to antimicrobial peptide resistance and outer membrane structural changes in Enterobacteriaceae and underproduction of OmpC leading to serum resistance in *Enterobacter* spp. concur with our previous observation of colistin-resistant *E. asburiae* and *K. pneumoniae* strains being tolerant to antimicrobial peptides [34].

This study identified OMP change as a possible mechanism for colistin resistance in *K. pneumoniae* ST258 and *E. asburiae*. Protein assortment change in the outer membrane may contribute to the stability and integrity of cell wall, thus developing and maintaining colistin resistance. Further investigations are required to explore the potentially elaborate regulatory system and the exact roles of the different proteins.

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### Conflict of Interest

The authors declare no conflict of interest.

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