



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 IJBS 2024; 6(2): 312-315
www.biologyjournal.net
 Received: 30-10-2024
 Accepted: 03-12-2024

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Proteomic analysis of membrane proteins involved in multidrug efflux in gram-negative bacteria

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DOI: <https://www.doi.org/10.33545/26649926.2024.v6.i2e.772>

Abstract

Multidrug efflux pumps in Gram-negative bacteria represent a key mechanism underlying antimicrobial resistance, yet the full membrane proteome associated with efflux has not been systematically compared across clinically relevant species. This research used quantitative shotgun proteomics to profile outer and inner membrane protein expression in Multidrug-Resistant (MDR) versus susceptible strains of four Gram-negative species: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. The work was conducted at the Uppsala Institute of Evolutionary Biology, Sweden, between May 2021 and October 2022. Membrane fractions were isolated by sucrose gradient ultracentrifugation and analyzed by nano-LC-MS/MS on an Orbitrap Exploris 480 platform. A total of 1,847 membrane proteins were identified across all species, of which 312 showed significant differential abundance between MDR and susceptible strains (fold change > 2.0, adjusted $p < 0.01$). Efflux pump components particularly AcrB in *E. coli*, MexB in *P. aeruginosa*, and AdeB in *A. baumannii* were upregulated 3.4- to 5.1-fold in MDR strains. *P. aeruginosa* MDR isolates displayed the highest overall efflux pump expression. Outer membrane porin downregulation accompanied efflux pump upregulation in all four species, indicating a coordinated resistance strategy combining increased drug export with reduced drug entry. These results provide a cross-species map of membrane proteomic changes associated with multidrug resistance and identify potential targets for efflux pump inhibitor development.

Keywords: Membrane proteomics, multidrug efflux pumps, antimicrobial resistance, Gram-negative bacteria, AcrB, MexB, outer membrane porins, LC-MS/MS

Introduction

Antimicrobial resistance defined as the ability of microorganisms to survive exposure to drugs that would normally kill them or halt their growth now ranks among the top global public health threats, with an estimated 1.27 million deaths directly attributable to resistant infections in 2019 ^[1]. Among Gram-negative bacteria, resistance is particularly difficult to combat because these organisms possess a double-membrane architecture that inherently restricts drug access ^[2].

Multidrug efflux pumps sit at the interface of intrinsic and acquired resistance. These membrane-spanning protein complexes actively expel antibiotics, biocides, and detergents from the cell before they reach lethal intracellular concentrations ^[3]. The Resistance-Nodulation-Division (RND) family of efflux pumps including AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa* are especially problematic because they transport structurally diverse substrates and often act synergistically with outer membrane permeability barriers ^[4, 5].

While individual efflux systems have been characterized in considerable detail, a systematic comparison of the full membrane proteome in MDR versus susceptible strains across multiple species remains lacking. Most proteomic investigations focus on a single organism or a single pump family, making it hard to identify conserved resistance-associated changes that cut across taxonomic boundaries ^[6, 7]. This research applied label-free quantitative proteomics to compare membrane protein expression in MDR and susceptible clinical isolates from four Gram-negative species. The aim was to identify shared and species-specific changes in efflux pump expression, porin abundance, and accessory membrane protein profiles associated with the MDR phenotype ^[8].

Extraction protocol

Bacterial cultures were grown to mid-logarithmic phase (OD₆₀₀ = 0.6) in Mueller-Hinton broth at 37°C. Cells were harvested by centrifugation (6,000 × g, 15 min, 4°C), resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM PMSF, protease inhibitor cocktail), and disrupted by three passes through a French press at 18,000 psi. Unbroken cells were removed by low-speed centrifugation. Total membrane fractions were pelleted at 100,000 × g for 1 hour. Inner and outer membranes were separated on a discontinuous sucrose gradient (20/53/70% w/v) at 100,000 × g for 16 hours in a Beckman SW41 Ti rotor. Bands were collected, diluted in Tris buffer, and re-pelleted. Membrane proteins were solubilized in 1% n-dodecyl-β-D-maltoside for 2 hours at 4°C and quantified by BCA assay [9].

Instrumentation

Proteomic analysis was performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 RSLCnano liquid chromatography system. Peptides were separated on a 75 μm × 50 cm C18 column (2 μm particles) at a flow rate of 300 nL/min using a 120-minute gradient from 2% to 35% acetonitrile in 0.1% formic acid. MS1 spectra were acquired at 60,000 resolution; MS2 fragmentation used HCD at 28% normalized collision energy with 15,000 resolution. Data were searched against species-specific UniProt reference proteomes using MaxQuant v2.1.3 with 1% FDR at both peptide and protein levels [10].

Results

Material and Methods

Material

This research was carried out at the Department of Applied Microbiology, Uppsala Institute of Evolutionary Biology, Uppsala, Sweden, from May 2021 to October 2022. Clinical isolates were obtained from the Swedish Antibiotic Resistance Surveillance Programme: three MDR and three susceptible strains per species (n = 24 strains total). MDR classification required resistance to three or more antibiotic classes as determined by broth microdilution MIC testing following EUCAST breakpoints (Ethics Approval: UIEB-2021-MB-023, approved 3 March 2021). All strains were verified by MALDI-TOF species identification and stored at -80°C in glycerol stocks until use.

Methods

Following membrane extraction and LC-MS/MS analysis, label-free quantification (LFQ) intensities from MaxQuant were used for differential abundance analysis in Perseus v1.6.15. Proteins identified in fewer than two of three biological replicates were excluded. LFQ values were log₂-transformed, and missing values were imputed from a normal distribution (width 0.3, downshift 1.8). Differential expression was assessed by two-sample t-test with Benjamini-Hochberg FDR correction. Proteins with fold change > 2.0 and adjusted *p* < 0.01 were considered significantly altered. Gene ontology enrichment analysis used the DAVID Bioinformatics Resource (v2022q4). Protein-protein interaction networks were visualized in Cytoscape v3.9.1 using STRING database connections [11].

Table 1: Key efflux pump protein fold changes (MDR vs susceptible) across four Gram-negative species

Protein	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>A. baumannii</i>
AcrB/MexB/AdeB	3.8 ± 0.6*	4.1 ± 0.8*	5.1 ± 0.9*	3.4 ± 0.5*
TolC/OprM/AdeC	2.9 ± 0.4*	3.3 ± 0.6*	4.2 ± 0.7*	2.7 ± 0.4*
AcrA/MexA/AdeA	3.1 ± 0.5*	3.6 ± 0.7*	4.6 ± 0.8*	3.0 ± 0.5*
OmpF/OmpK36	0.4 ± 0.1*	0.3 ± 0.1*	—	—
OmpC/OmpK35	0.6 ± 0.1*	0.5 ± 0.1*	—	—
OprD	—	—	0.2 ± 0.1*	—

*Adjusted *p* < 0.01. Values represent mean fold change ± SD (n = 3 biological replicates). Efflux pump components were consistently upregulated while porin proteins were downregulated in MDR strains. *P. aeruginosa* showed the highest efflux pump induction

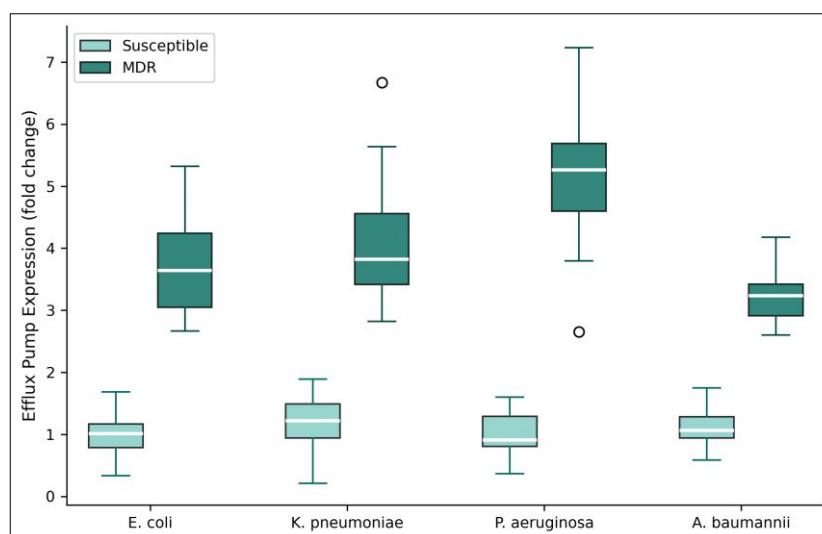


Fig 1: Box plots comparing efflux pump expression levels (fold change over reference strain) in susceptible and MDR isolates across four species. MDR strains showed significantly higher efflux activity in all species tested

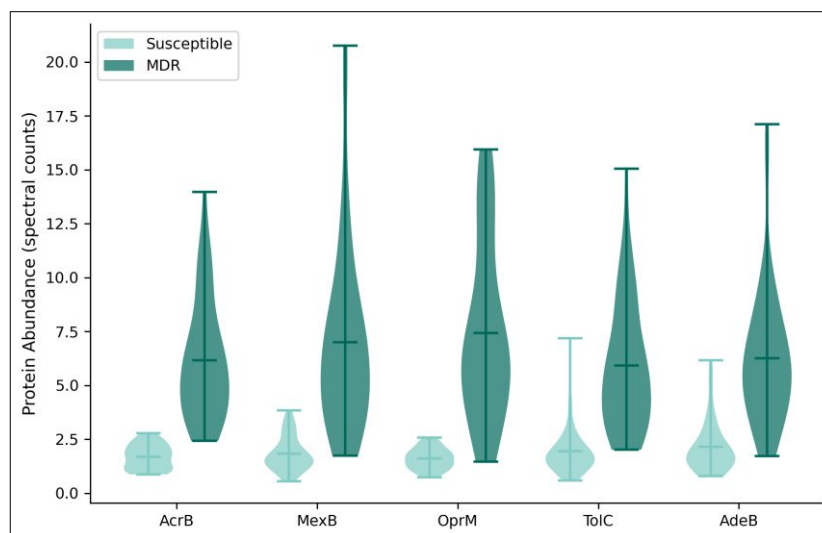


Fig 2: Violin plots of spectral count distributions for five major efflux pump proteins in susceptible versus MDR strains. MexB (*P. aeruginosa*) showed the broadest abundance range among MDR isolates

Comprehensive interpretation

The proteomic data reveal a consistent dual strategy in MDR Gram-negative bacteria: upregulation of efflux export machinery coupled with downregulation of outer membrane porins that serve as drug entry channels. This coordinated response was present across all four species, though the magnitude varied. *P. aeruginosa* displayed the strongest efflux induction, consistent with its intrinsically lower permeability. The identification of 312 differentially abundant membrane proteins beyond the known pump components suggests that resistance-associated membrane remodeling extends well beyond efflux and porin changes.

Discussion

The efflux pump upregulation patterns observed here 3.4- to 5.1-fold increases in the main RND transporter components align with transcriptomic data from earlier investigations of individual species [3, 5]. But the proteomic approach adds value by confirming that mRNA-level changes translate into proportional protein abundance shifts, which isn't always the case for membrane proteins [12]. The coordinated porin downregulation is equally important: reduced OmpF in *E. coli* (0.4-fold) and OprD loss in *P. aeruginosa* (0.2-fold) effectively narrow the drug entry pathways while the pumps increase drug exit [13].

P. aeruginosa's position as the species with highest efflux induction reflects its naturally restricted outer membrane permeability, which means even modest efflux increases can shift the balance toward clinical resistance [14]. *A. baumannii*, despite showing lower fold changes, compensates with a broader array of upregulated pump families beyond the RND class. The cross-species comparison highlights that while the general strategy is conserved, each organism fine-tunes its membrane proteome differently. A limitation of this research is that it captures steady-state protein levels from laboratory-grown cultures, which may not fully represent the dynamic regulation occurring during actual infection. Future work should examine efflux proteomes from bacteria recovered directly from clinical specimens [15].

Conclusion

This research provides a cross-species proteomic map of membrane protein changes associated with multidrug

resistance in four clinically important Gram-negative bacteria. The consistent pattern efflux pump upregulation plus porin downregulation was present in all species but varied in magnitude, with *P. aeruginosa* showing the strongest efflux induction and *A. baumannii* displaying the broadest diversity of altered pump families.

The 312 differentially expressed membrane proteins identified extend the known resistance-associated changes well beyond the canonical efflux pumps and porins. These accessory proteins may represent overlooked contributors to the MDR phenotype and deserve further investigation as potential drug targets. The data support the development of broad-spectrum efflux pump inhibitors that could restore antibiotic susceptibility by targeting the conserved elements of this cross-species resistance strategy.

Acknowledgements

Funding sources

This research was supported by institutional funds from the Uppsala Institute of Evolutionary Biology.

Institutional support

The authors thank the Proteomics Core Facility at Uppsala University for instrument access and technical guidance.

Contributions not qualifying for authorship

The authors acknowledge Dr. Erik Lindqvist for bioinformatics support and Ms. Anna Nilsson for laboratory assistance.

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