VILNIUS UNIVERSITY

Jūratė
SKERNIŠKYTĖ

# Molecular mechanisms of *Acinetobacter baumannii* pathogenesis

**SUMMARY OF DOCTORAL DISSERTATION**

Natural sciences
Biochemistry N 004

VILNIUS 2019

The dissertation was written between 2014 and 2018 in Vilnius University Life Sciences Center Institute of Biosciences.

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This doctoral dissertation will be defended in a public meeting of the Dissertation Defense Panel:
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The dissertation shall be defended at a public meeting of the Dissertation Defense Panel at 10.00 am on 27 September 2019 in R108 auditorium of the Life Sciences Center (Vilnius University).

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VILNIAUS UNIVERSITETAS

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# *Acinetobacter baumannii* patogenezės molekuliniai mechanizmai

**DAKTARO DISERTACIJOS SANTRAUKA**

Gamtos mokslai
Biochemija N 004

VILNIUS 2019

Disertacija rengta 2014–2018 metais Vilniaus universiteto Gyvybės mokslų centro Biomokslų institute

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Disertacija ginama viešame Gynimo tarybos posėdyje 2019 m. rugsėjo mėn. 27 d. 10.00 val. Gyvybės mokslų centre (Vilniaus universitetas) R108 auditorijoje. Adresas: Saulėtekio al. 7, LT- 10257 Vilnius, Lietuva.

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje ir VU interneto svetainėje adresu: *https://www.vu.lt/naujienos/ivykiu-kalendorius*

# INTRODUCTION

Gram-negative bacterium *Acinetobacter baumannii* is a difficult to treat infection agent, causing nosocomial infections worldwide (Holt *et al*., 2016). Characteristic features of this opportunistic pathogen include multidrug-resistance (MDR) phenotype, ability to withstand unfavorable environmental conditions for long periods of time and a high propensity for spread resulting in the hospital outbreaks, especially in the intensive care units (Eijkelkamp *et al*., 2014). *A. baumannii* demonstrates the highest resistance rates among hospital-associated MDR bacteria during the last ten years (Giammanco *et al*., 2017). The worldwide spread of *A. baumannii* in clinical settings is characterized by the expansion of a several predominant clones (Karah *et al*., 2011; Zarrilli *et al*., 2013). Of them, the international clonal lineages I (IC I) and II (IC II) account for the most part of the *A. baumannii* infections (Zarrilli *et al*., 2013; Dahdouh *et al*., 2017).

*A. baumannii* demonstrates high genome plasticity (Imperi *et al*., 2011; Snitkin *et al*., 2011; Eijkelkamp *et al*., 2014). Strains belonging to distinct IC harbor unique sets of genes or their variants, that impact virulence-associated features of *A. baumannii* strains (Eijkelkamp *et al*., 2014). In order to persist in clinical settings *A. baumannii* must be equipped with a set of cell surface features enabling it to adhere to the abiotic surfaces found in medical devices as well as to survive under desiccation stress (Greene et al., 2016; Chiang *et al*., 2018).

*A. baumannii* has been considered as a top priority pathogen for which new drugs and therapeutic options are urgently needed (Isler *et al*., 2018). Among strategies considered, the vaccine-based approaches show the greatest potential (Dickey, 2017). The desirable antigens should be highly prevalent surface-exposed bacterial proteins and possess high degree of conservation among clinical strains. Therefore, long bacterial adhesins are of increasing interest

for the development of new vaccine candidates (Badmasti *et al*., 2015; Sheel *et al*., 2016).

This thesis includes investigation of virulent features and surface-related cellular components of clinical *A. baumannii* strains from Lithuanian hospitals, and studies on the characterization of new vaccine candidates against *A. baumannii*.

**The aim of this thesis** was to study the properties of clinical *A. baumannii* strains and the molecular components relevant to the virulence of bacteria.

Towards this goal, the following **tasks** were formulated:

- Identify the phenotypic properties of pandemic *A. baumannii* strains and reveal the links with the virulence;
- Determine the significance of *A. baumannii* surface components for the virulence-associated bacterial properties;
- Investigate *A. baumannii* surface-exposed Blp1 protein as potential vaccine candidate.

**Scientific novelty**

For the first time here we represent comprehensive investigation of the cell surface-related phenotypic properties of pandemic *A. baumannii* clinical strains isolated from Lithuanian hospitals. We have identified their relations with the virulence, since specific clone-associated features are largely obscure. We have found that the phenotypes of bacteria movement and pellicle formation are unique to *A. baumannii* strains belonging to IC I (international clone I). We also showed for the first time that pandemic strains differ in their profiles of the capsular polysaccharides.

In this work we have shown that *A. baumannii* strains belonging to IC II are distinguished by the cell surface hydrophilicity, which determines the different virulent properties of these strains. Hydrophilic strains exhibit more pronounced resistance to dryness, adhesion to eukaryotic cells, produce a thicker cell wall structure and are more virulent *in vivo*, but at the same time, form a thinner biofilm on the plastic surface compared to hydrophobic strains. We also found that the ability of *A. baumannii* strains to form the biofilm correlated with their prevalence in hospitals. We have identified DNA sequences of *A. baumannii* strains with hydrophobic and hydrophilic properties and have found the correlation between the hydrophobic properties of IC II strains and the carriage of pACICU2-type plasmid.

We have identified YgaU and GltI proteins as new *A. baumannii* virulence factors. We have determined the relationship of the allelic variants of *ompA* and *blp1* gene on the clonality of the strains. For the first time we have shown that OmpA interaction with the peptidoglycan is an important factor in maintaining the integrity of bacterial cell wall and in the process of cell division. Moreover, the interaction of the OmpA protein with peptidoglycan affected multiple virulence-associated features of *A. baumannii*. We described the effect of Blp1 protein on adhesion of IC I and IC II strains to the plastic and eukaryotic cells. We demonstrated the importance of Blp1 protein during the infection *in vivo*.

Using the mice sepsis model during active and passive immunization, we described a fragment of C-terminus of Blp1 protein as a new vaccine candidate against infections caused by clinical *A. baumannii* strains. We have shown that the Blp1 fragment ensures more efficient effect compared to the OmpA protein in both serum-induced opsophagocytosis as well as in the evaluation of mouse survival during active immunization.

**Major findings presented for the defense of this thesis:**

1. Pandemic *A. baumannii* strains differ in their virulent properties;
2. The hydrophilicity of *A. baumannii* surface results in increased virulence of the bacteria, but is not a favorable property for the spread of clinical strains;
3. *A. baumannii* OmpA, Blp1, YgaU and GltI proteins are virulence factors *ex vivo* and *in vivo*;
4. The multifunctional role of OmpA in *A. baumannii* pathogenesis is determined by the interaction of periplasmic domain of the protein with peptidoglycan;
5. C-terminal fragment of *A. baumannii* Blp1 protein is an effective antigen for active immunization, while the Blp1-antiserum effectively protects against *A. baumannii* infection during the passive immunization.

**The thesis contents**

This thesis is written in Lithuanian and contains the following parts: Abbreviations, Introduction, Scientific Novelty, Literature Review, Materials and Methods, Results, Discussion, Conclusions, List of Publications within the area of the thesis (2 publications, 6 conference presentations), Acknowledgements, References (281 citations), Supplements (2 Figures). Thesis includes Figures (36), Tables (5). Total 128 pages.

**Table 1.** Primers, plasmids and strains used in this study

| Primer | Sequence (5'- 3') | Purpose | Source |
|---|---|---|---|
| Fjgms | AGGACAGAAATGCCTCGAC | Amplification of *aac(3)-I* | This work |
| Rjgms | ATCTCGGCTTGAACGAATT | | |
| P-Ab-ITSF | CATTATCACGGTAATTAGTG | Detection of *A. baumannii* species | Chiang *et al*., 2011 |
| P-Ab-ITSB | AGAGCACTGTGCACTTAAG | | |
| gnaaF | CNTAYTAYYTDACNCATAAAGC | Amplification of K locus | This work |
| galuR | GTCAACNACBGTDACCATTTC | | |
| wzy11F | AACGTTGGGACTATAGCAACAAAT | Identification of *wzy11* gene and KL2 type | This work |
| wzy11R | CCTGTTTGATGGGGTGGTCT | | |
| RH1704 | CCCTACAAGGTCTTGCCAAT | Identification of OCL1 | Kenyon et al., 2014 |
| RH1705 | CCTCAGCCCGTACTTACAAC | | |
| Bav1 | CATTACAATGCTTAAGCTA | Amplification of upstream region of *blp1* | This work |
| Bav2 | CATATTAAACAATAATTTGCTTCCTTCAT CGGTAGAAAC | | |
| Bav3 | GCAAATTATTGTTTAATATG | Amplification of downstream region of *blp1* | This work |
| Bav4 | GTCGAGGCATTTCTGTCCTGGTTTAGCAA TAGAACGGAT | | |
| OmpA1 | GGAGCAGTTAGTCCTGATAG | Amplification of upstream region of *ompA* | This work |
| OmpA2 | GAACTCAAATTATTGAGCTGCCTCCAGAG ATAACAATTG | | |
| OmpA3 | CAGCTCAATAATTTGAGTTC | Amplification of downstream region of *ompA* | This work |
| OmpA4 | GTCGAGGCATTTCTGTCCTTGCTTCGTCA GTTTGAGGC | | |
| OAsp268F | CACACAGCTAACACTGGTCCACGTAAG | Site-directed mutagenesis of Asp268 to Ala in *ompA* gene | This work |
| OAsp268R | GACCAGTGTTAGCTGTGTGACCTTCG | | |
| OmklF | CCTTAATATATGTATAAATAGAGC | Amplification of *ompA* gene with upstream region | This work |
| OmklR2 | AGTCGGTACCAGATTATGAATCAGGAGA TTTAC | | |
| Aac3I_seqR | CGAAGTCGAGGCATTTCTGT | Confirmation of transformants | Laboratory collection |
| AcORI_seqR | AGGCTGTTGATAACTTTTGGAA | | |
| BldBamF | TTCTAGGATCCGAATATTGCTCCAGTAAT T | Cloning of $blp1_{2652\text{-}3363}$ fragment into expression vector | This work |
| BlXhR | TTCTACTCGAGTTAAACAATAATTTGCTG G | | |
| OmBamF | TTCTAGGATCCGATGAAATTGAGTCGTAT T | Cloning of *ompA* into expression vector | This work |
| OmXhR | TTCTACTCGAGTTATTGAGCTGCTGCAG | | |
| Sel1 | CTTGCTACACTCATGTTGA | Amplification of upstream region of *sel1* | This work |
| Sel2 | CAGCTTGTTTATTCTTCTCAGCTTGAAAT GGCAATTAGACTG | | |
| Sel3 | CTGAGAAGAATAAACAAGCTG | Amplification of downstream region of *sel1* | This work |
| Sel4 | GTCGAGGCATTTCTGTCCTTAAACTGGTA ACACCTGAAC | | |
| Lys1 | CTCTTTTACGCCGAATTCAG | Amplification of upstream region of *ygaU* | This work |
| Lys2 | CATTAAATTGAGGTGGCTGACATGAAAA TTAACCATGCCGTTA | | |
| Lys3 | TCAGCCACCTCAATTTAATG | Amplification of downstream region of *ygaU* | This work |
| Lys4 | GTCGAGGCATTTCTGTCCTTGGAGTATTC GTATTAGCTTC | | |
| Peb1 | GATAGTTTGGCAGATGAACTT | Amplification of upstream region of *gltI* | This work |
| Peb2 | AGATCCAGTTAGATTCCTGCTTCATAAGA ACTCCTTGATGATC | | |
| Peb3 | GCAGGAATCTAACTGGATCT | Amplification of downstream region of *gltI* | This work |
| Peb4 | GTCGAGGCATTTCTGTCCTTGATTAAATA GACAGGTGTAGAC | | |
| Brk1 | CGTTCAATCACTTGCTGA | Amplification of upstream | This work |

| | | | |
|---|---|---|---|
| Brk2 | TGTTGAAAGTGGAATAGAGAGTTCTAAC ATGCGTCATCTATC | region of *brkB* | |
| Brk3<br>Brk4 | CTCTCTATTCCACTTTCAACA<br>GTCGAGGCATTTCTGTCCTGGTGCTAAAT GGAAGAGC | Amplification of downstream region of *brkB* | This work |
| VP1<br>VP2 | CTTCCTGATTGGAATTCTGG<br>ATAGTCTGCCATTAACTGTAATTCAGACA TACTCATTGTTCT | Amplification of upstream region of *pqiB* | This work |
| VP3<br>VP4 | TTACAGTTAATGGCAGACTAT<br>GTCGAGGCATTTCTGTCCTTGAAACTCAA CCAACAAGT | Amplification of downstream region of *pqiB* | This work |

| **Plasmids** | **Characteristics** | **Source** |
|---|---|---|
| pUC19_sacB | pUC19 derivative with *sacB* gene from *Bacillus* sp.; for the generation of markerless gene deletion mutants | Laboratory collection |
| pUC19_gm_AcORI (p) | pUC19 derivative with *aac(3)-I* gentamicin aminoglycoside acetyltransferase cassette and *ori* site from *Acinetobacter* spp.; for the complementation experiments | Laboratory collection |
| p*ompA* | pUC19_gm_AcORI derivative with *ompA* gene along with upstream region (putative promoter) from $Ab_{IC\ I}$ strain; for the complementation experiments | This work |
| p$ompA_{D268A}$ | pUC19_gm_AcORI derivative with *ompA* gene with D268A substitution along with upstream region (putative promoter) from $Ab_{IC\ I}$ strain; for the complementation experiments | This work |
| pET-28b | Protein expression vector | Novagen |
| pET-His-OmpA | OmpA expression plasmid, His-tag fused N-terminally to protein | This work |
| pET-His- $Blp1_{2652-3363}$ | Blp12652-3363 expression plasmid, His-tag fused N-terminally to protein | This work |

| **Strains** | **Characteristics** | **Source** |
|---|---|---|
| *Escherichia coli* OP50 | Wild type, bacterial food source for *C. elegans* | Brenner, 1974 |
| *Acinetobacter baylyi* ADP1 | Wild type | ATCC 33305* |
| *Acinetobacter baumannii* $Ab_{IC\ I}$ | Representative IC I clone strain; MDR strain, gentamicin sensitive | Povilonis *et al.*, 2013 |
| $Ab_{IC\ I}$*ΔompA* | *ompA* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |
| $Ab_{IC\ I}$*ΔompA*::p | $Ab_{IC\ I}$Δ*ompA* strain with pUC19_gm_AcORI plasmid | This work |
| $Ab_{IC\ I}$*ΔompA*::p*ompA* | $Ab_{IC\ I}$Δ*ompA* strain complemented with p*ompA* | This work |
| $Ab_{IC\ I}$*ΔompA*::p$ompA_{IC\ II}$ | $Ab_{IC\ I}$Δ*ompA* strain complemented with p$ompA_{IC\ II}$ | This work |
| $Ab_{IC\ I}$*ΔompA*::p$ompA_{D268A}$ | $Ab_{IC\ I}$Δ*ompA* strain complemented with p$ompA_{D268A}$ | This work |
| *E. coli* JM107 | endA1 glnV44 thi-1 relA1 gyrA96 Δ(lac-proAB) [F_ traD36 proAB+ lacIq lacZΔM15] hsdR17(RK- mK+) λ- | Yanisch-Perron *et al.*, 1985 |
| *E. coli* BL21 (DE3) | ompTgaldcmlonhsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07ind1sam7nin5]) [malB+]K-12(λS) | Studier and Moffatt, 1986 |
| *E. coli* ArcticExpress (DE3) | B $F^-$ ompT hsdS($r_B^-$ $m_B^-$) $dcm^+$ $Tet^r$ gal λ(DE3) endA Hte [cpn10 cpn60 $Gent^r$] | Thermo Fisher Scientific |
| $Ab_{IC\ I}$*Δsel1* | *sel1* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |
| $Ab_{IC\ I}$*ΔygaU* | *ygaU* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |
| $Ab_{IC\ I}$*ΔgltI* | *gltI* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |
| $Ab_{IC\ I}$*ΔbrkB* | *brkB* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |
| $Ab_{IC\ I}$*ΔpqiB* | *pqiB* gene-negative mutant of *A. baumannii* strain $Ab_{IC\ I}$; markerless | This work |

***A. baumannii* growth assays**

Bacterial growth was evaluated in LB medium, 80% active fetal bovine serum (FBS) and 80% heat inactivated FBS (htFBS) as described by Skerniškytė *et al.* (2019). Growth was measured at 37°C with periodic shacking every 20 min by Tecan Infinite M200 Pro microplate reader.

**Determination of cell surface hydrophobicity**

Determination of cell surface hydrophobicity by Salt Aggregation Test (SAT) was carried out as described by Nwanyanwu and Abu (2013). Briefly, *A. baumannii* were grown on LB plates at 37°C overnight. Cells were suspended in dd$H_2O$ until slight turbidity and mixed with the equal volume of ammonium sulfate solution to yield concentrations ranging from 0.0625 M to 2 M. Cell aggregation (climbing) was observed under the microscope at 50 × magnification. The cell surface hydrophobicity was expressed as a lowest salt concentration, which caused bacterial cell aggregation.

**Generation of deletion mutants, complemented strains and site-directed mutagenesis**

Markerless gene deletion was performed as previously described (Oh *et al*., 2015). Plasmids used for mutant generation are listed in Table 1. Mutants were selected by PCR with specific primers and confirmed by sequencing. For the complementation, the sequence of genes with upstream region (with the potential native promoter sequences) was amplified using primers listed in Table 1 and cloned into pUC19_gm_AcORI plasmid. Site-directed mutagenesis of OmpA-like domain was performed using inverse PCR with primers OAsp268F/OAsp268R and p*ompA* plasmid as a template (Table 1). All generated plasmids were confirmed by sequencing. *ompA* gene deletion mutant was transformed with the resulting plasmids by electroporation and colonies were selected on LB agar with 30 μg/ml gentamicin.

**Confocal Laser Scanning Microscopy (CLSM)**

For evaluation of biofilms, 1000-fold dilutions of overnight *A. baumannii* cultures were used for seeding into LB media. Biofilms were grown for 2 and 24 h at 37°C without agitation. After growth in micro-titer plates, biofilms were stained for 2 h by the Filmtracer TM LIVE/DEAD® Biofilm Viability Kit (Thermo Fisher Scientific). The plate was then placed on the motorized stage of an inverted confocal microscope (TCS SP8 AOBS, Leica Microsystems) at the INRA-MIMA2 imaging platform (Jouy en Josas, France) as described by Poquet *et al.* (2018).

**Transmission electron microscopy**

TEM analysis was undertaken at Microscopy and Imaging Platform MIMA2 at Gabi UMR (Jouy en Josas, France). Bacteria were grown in LB medium at 37°C

overnight and cells were fixed within 0.1 M sodium cacodylate buffer (pH 7.2) with 2% of glutaraldehyde for 3 hours at room temperature. After treatment with 0.5% Oolong Tea Extract (OTE) in cacodylate buffer, post-fixation with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, pellets were dehydrated in solutions of increasing ethanol concentrations and embedded in Epon. Ultrathin sections were collected on 200-mesh copper grids and counterstained with lead citrate. Grids were examined with a Hitachi HT7700 electron microscope operated at 80 kV (Elexience, France), and images were acquired with a charge-coupled device camera (AMT).

**Cell culture assays**

Mouse epithelial LL/2 (LLC1) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C with 5% $CO_2$. Adhesion experiments were performed as described by Skerniškytė *et al*. (2019). Bacterial adherence (A%) to the LL/2 cells was expressed as a percentage of the CFU of adhered bacteria compared to the total number of CFUs of the initial inoculum.

For the opsonophagocytic killing assay, J774 macrophages were grown in DMEM supplemented with 10% FBS at 37°C with 5% $CO_2$. Macrophages were stimulated with 40 ng/ml *E. coli* LPS (Sigma-Aldrich) for 3 days. The macrophages ($1 \times 10^5$ cells/well) and *A. baumannii* strains ($1 \times 10^4$ CFU/well) were added into the wells along with the heat-treated mice serum (56 °C for 40 min for the inactivation of complement components) at the final dilution of 1:200. After 1 h of incubation the samples were serially diluted and seeded. Serum killing rates were counted by comparing the number of the reduced CFUs with those observed using naïve serum.

A cytotoxicity assay was performed by incubating LL/2 with recombinant Blp1 C-terminal fragment and OmpA protein in DMEM supplemented with 10% FBS at 37°C for 24 hours. Then, 20 µl of MTS solution was added to the wells and cells were incubated for 4 hours at 37 °C. After incubation $OD_{490}$ was determined. The proliferation rate was counted by comparing $OD_{490}$ values for antigen-treated and non-treated cells.

***Caenorhabditis elegans* fertility assay**

*A. baumannii* strains were investigated using *C. elegans* fertility model as described by Skerniškytė *et al*. (2019). Overnight cultures of different *A. baumannii* strains were seeded on NGM medium. One L2 stage worm was placed over each *A. baumannii* strain. On the third day after infection worm progeny was determined by counting *C. elegans* worms.

**Cloning and proteins purification**

The DNA of Blp1 C-terminal fragment spanning 2652-3363 amino acids of *blp1* coding region and full coding region of *ompA* with 6×His tag sequences, attached to the N-terminus of the recombinant proteins were amplified using primer pair BldBamF/BlXhR and OmBamF/OmXhR, respectively. The resulting amplicon was cloned into a protein expression plasmid pET-28b. The resulting plasmids were sequenced and transformed into the expression host strain *E. coli* BL21 cells for OmpA expression and for $Blp1_{2652-3363}$ expression the host strain *E. coli* ArcticExpress was used. Culture was grown in Luria-Bertani (LB) broth containing 40 μg/ml of kanamycin to OD600 of 0.5. Protein expression was induced using 0.5 mM isopropyl β-d-thiogalactopyranoside (IPTG) at 28°C for OmpA expresson and at 14°C for $Blp1_{2652-3363}$ expression for 16 h. Cells were harvested by centrifugation at 7000 rpm for 15 minutes and cell pellets were suspended in lysis buffer (20 mM $NaH_2PO_4$ pH 7.4, 500 mM NaCl, 20 mM imidazole) supplemented with protease inhibitor PMSF. Cells were disrupted by sonication and centrifuged 12000 rpm at 4ºC, for 30 min to collect insoluble material. Proteins were purified from soluble fraction by affinity chromatography, using 1 ml HisTrapHPTM nickel-Sepharose column (GE Healthcare). Proteins were eluted by linear gradient using buffer containing 20 mM $NaH_2PO_4$ pH 7.4, 500 mM NaCl and 500 mM imidazole. The eluted fractions were desalted using Sephadex G–25 (GE Healthcare) column, exchanging to PBS buffer. Eluted proteins were analyzed by 12% SDS–PAGE gels, stained with Coomassie Brilliant Blue. The final recombinant protein product was frozen and stored at −80°C for further studies.

**Murine models**

Eight- to twelve-week-old female BALB/c mice were purchased from Institute of Biochemistry, Life Science Center (Vilnius University, Vilnius). The animals were maintained and used in accordance with the recommendations of the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purpose. Study was performed under permission of Lithuanian State Food and Veterinary Service no. G2-72.

A sepsis model was established as described previously (Skerniškytė *et al.*, 2019). Briefly, *A. baumannii* cultures were prepared by mixing the bacterial suspension with 5% of porcine mucin (w/v; Sigma-Aldrich). BALB/c mice were injected intraperitoneally with 0.5 ml of the sample. The CFUs corresponding the bacterial loads were determined by plating sequential dilutions on LB plates.

For the immunization experiments, groups of BALB/c mice (n = 5) were immunized intramuscularly with 2 μg of recombinant Blp1 protein fragment or OmpA protein. Immunization mixture was prepared by mixing the antigen with an equal volume of complete Freund's adjuvant on the day 0 and with incomplete Freund's adjuvant on the days 14 and 28. Control group was inoculated with PBS

combined with Freund's adjuvant. On the day 32, blood samples were collected and tested against immunogen using ELISA. Mice were challenged intraperitoneally on the day 42 with $1 \times 10^8$ CFU of $Ab_{IC\ I}$ bacteria. For the passive immunization, the 200 μL of antiserum was injected intraperitoneally into naïve mice (3 mice per group). Control group received the serum obtained from mice, immunized with PBS and Freund's adjuvant. After 6 hours, $5 \times 10^7$ CFU of $Ab_{IC\ I}$ bacteria were injected intraperitoneally and mice viability was monitored for 45 days.

**Statistical analysis**

All statistical comparisons were based on t-test or the one-way analysis of variance (ANOVA) with a Tukey HSD post hoc test.

# RESULTS AND DISCUSSION

This dissertation is divided into three major parts. The first part represents a comprehensive characterization of virulence-related traits of clinical *A. baumannii* strains isolated from Lithuanian hospitals. All tested strains belonged to the most prevalent *A. baumannii* clonal lineages, namely international clone I (IC I) and international clone II (IC II). The second part describes the investigations of potential virulent factors of *A. baumannii*. Finally, in the last part, two major virulent surface-exposed proteins Blp1 and OmpA were characterized as vaccine candidates against clinical *A. baumannii* strains using murine infection model.

## Virulence-related features of clinical *A. baumannii* strains

Thirty six *A. baumannii* clinical isolates, chosen for the present study, were representatives of 30 distinct PFGE types (pulsotypes) of IC I and IC II clonal lineages, and were obtained from Lithuanian hospitals during the period of June-November 2010 (Povilonis *et al*., 2013). Twenty IC I isolates and sixteen IC II isolates were selected. Most of the isolates tested belonged to the prevalent pulsotypes (more than two strains in pulsotype), while some of the isolates were sporadic (two or one strain in the pulsotype). All isolates were multidrug-resistant (resistant to three or more antibiotic classes).

We were interested, whether representative strains of the two most common clonal lineages display specific pattern of surface-related features, which are thought to be important for *A. baumannii* growth and survival in clinical environment and within the host (Rumbo *et al*., 2014; Weber *et al*., 2015; Lee *et al*., 2017). Therefore, we first tested swarming and twitching motilities of selected *A. baumannii* isolates. The swarming distance, expressed by the majority of strains (92%, 33/36), was low and yielded approximately 6 – 14 mm. Only three strains, all representatives of IC I lineage,

showed increased swarming motility yielding a value of > 26 mm. (Figure 1A). However, the majority of *A. baumannii* IC I lineage strains showed twitching motility in contrast to IC II strains, which lacked this property with the exception of a single strain (Figure 1A). The IV type pili have been proposed to be responsible for twitching motility in *A. baumannii* (Harding *et al*., 2013), therefore we looked for the presence of pili-like structures on the cell surface. The transmission electron microscopy of representative motile IC I strain $Ab_{IC\ I}$ and non-motile IC II strain II-a showed marked differences in cell surface structures, the IC I strain displaying pili-like extended structures, which were absent in the IC II strain (Figure 1B).

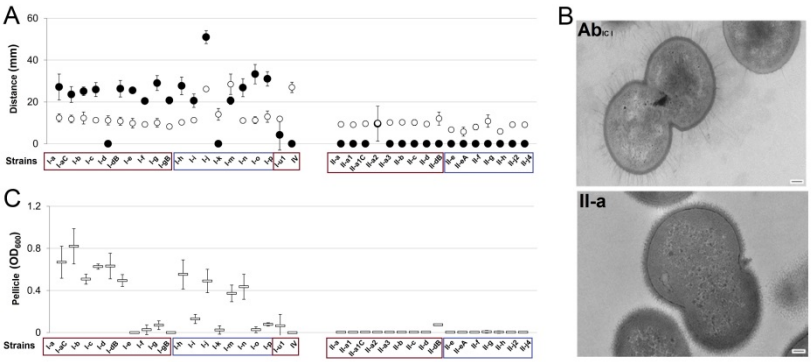


**Fig. 1.** Twitching and swarming motilities and pellicle formation of *A. baumannii* IC I and IC II lineage strains. A – twitching (●) and swarming (o) motilities expressed as a distance in mm; B – TEM analysis of $Ab_{IC\ I}$ and II-a strains ; C – total pellicle biomass (═) was suspended in the aqueous solution and absorbance $OD_{600}$ was measured. Data are given as mean ± standard deviations from three independent experiments. Roman numerals I and II in the strains names indicate IC I and IC II, respectively. Red boxes indicate prevalent strains, while blue boxes present sporadic strains.

According to the recent observations, the bacterial motility contributes to the formation of pellicle, a biofilm at the air-liquid interface (Hölscher *et al*., 2015; Giles *et al*., 2015). Hence, we tested the ability of our set of *A. baumannii* strains to form pellicle by growing them in TSB medium. The vast majority of IC II lineage strains lacked the ability to form pellicle, whereas 85% (17/20) of IC I strains showed pellicle-forming phenotype (Figure 1C). The pellicle formation clearly was a trait of IC I lineage, though these

strains were highly various in terms of the abundance of pellicle biomass. However, there was no obvious correlation among pellicle formation and swarming or twitching motility in IC I group, as for example I-d strain lacking twitching motility phenotype, was able to form a pellicle and I-gB strain with no pellicle forming feature was able to demonstrate twitching motility. In accordance with other studies (Eijkelkamp *et al*., 2011), our results show that swarming hypermotility is a rare phenotype, as we have identified only three strains with this type of motility. Moreover, our results indicate that twitching motility and pellicle formation are features strongly associated with *A. baumannii* IC I lineage strains, however, these characteristics had no correlation with the prevalence of the strains. These data are in the line with the previous observations of Eijkelkamp *et al*. (2011) from the analysis of Australian *A. baumannii* clinical isolates, where all IC I clone and only few IC II clone members showed twitching motility.

Similarly to other Gram-negative pathogens, *A. baumannii* capsular polysaccharide (CPS) and lipopolysaccharide (LPS), the latter thought to be deficient in extracellular polysaccharide portion in most *A. baumannii* strains (Harding *et al*., 2015) and called lipooligosaccharide (LOS), are essential virulence factors protecting from the host complement system (Russo et al., 2010) and mediating inflammatory responses (Moffat *et al*., 2013). Figure 2 shows polysaccharide profiles of representative IC I (n=5) and IC II (n =11) isolates. Major differences can be observed in CPS profiles between representatives of two clonal lineages. The IC I strains express CPS of variable length, whereas the IC II strains with the few exceptions, produce only narrow distribution, high-molecular-weight CPS. Two IC II strains (II-h and II-dB) were found to be capsule-deficient at growth conditions used (Figure 2). Our data show that nearly all clinical *A. baumannii* strains produce capsule, although IC I and IC II strains display lineage-specific CPS profile. According to the previous reports, *A. baumannii* genomic K locus, responsible for the

CPS synthesis and export, is highly diverse (Hu *et al*., 2013; Kenyon and Hall, 2013). Strikingly, all our examined capsule-producing IC I strains synthesized polysaccharides of variable-molecular-mass, whereas IC II strains yielded exclusively high-molecular-mass CPS.

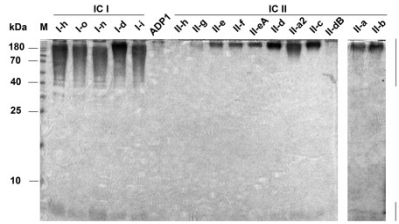


**Fig. 2.** Capsular polysaccharide (CPS) profiles of *A. baumannii* IC I and IC II lineage strains. 12% SDS-PAGE followed by Alcian Blue staining was undertaken to visualize CPS. *Acinetobacter baylyi* strain ADP1 was used as CPS-negative control. LOS denotes lipooligosaccharide. The positions of standard molecular mass markers are shown on the left.

One of the most important characteristics of bacterial surface is cell hydrophobicity, whereas it plays a role in virulence-associated processes (Krasowska and Sigler, 2014). All our tested IC I strains were considered to have hydrophobic character based on the estimated Salt Aggregation Test (SAT) values, which ranged from 0.5 M to 1 M (Figure 3A). In contrast, more than a half (56%, 9/16) of IC II strains displayed low surface hydrophobicity compared with the IC I group (SAT values ≥2 M) (Figure 3A). Of hydrophilic IC II strains, II-a2, II-c, II-d and II-dB represented clonal isolates, with strains belonging to the pulsotypes retrieved repeatedly from the hospitals, whereas II-e, II-eA, II-f, II-g, II-h isolates were sporadic. We did not observe a correlation between hydrophobicity and sequence type (ST) by examining selected IC II isolates according the Oxford multilocus sequence typing (MLST) scheme (https://pubmlst.org/abaumannii/). Thus, strains II-a and II-f, differing significantly in hydrophobic features, were both assigned to a common sequence type ST208, whereas strains II-a2 and II-h displaying hydrophilic character were assigned to different STs, ST 440 and ST348, respectively.

Since, it has been proposed that a degree of cell surface hydrophobicity could modulate adhesive properties of various commensal and pathogenic microorganisms (Krasowska and Sigler, 2014), we investigated how hydrophobic character of *A. baumannii* IC I and IC II strains impacts their ability to form biofilms and adhere to the abiotic and biotic surfaces. Interestingly, the trend of biofilm formation among IC I and IC II strains was different to that observed for pellicle phenotype (Figure 3B).

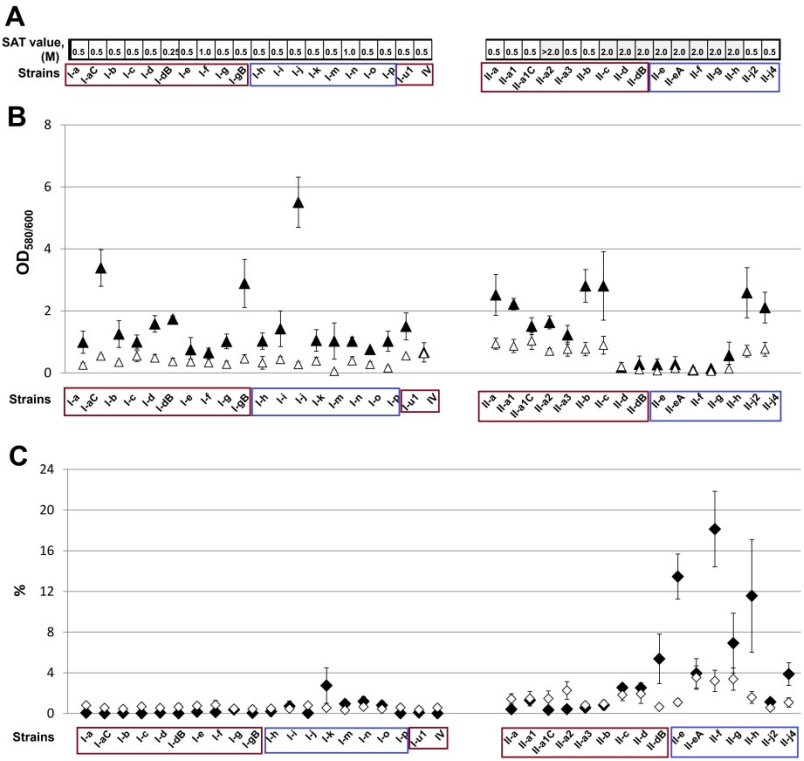


**Fig. 3.** The cell surface-associated features of *A. baumannii* IC I and IC II lineage strains.
A – surface hydrophobicity, defined by the salt aggregation test (SAT) and expressed as a minimum ammonium sulfate concentration (M) required for bacterial aggregation; B – biofilm formation (▲) and adherence to polystyrene (Δ) expressed as $OD_{580/600}$. Error bars represent standard deviations from three independent experiments; C –desiccation resistance (♦) and adhesion to lung epithelium cells LL/2 (◊) expressed as percentages. Error bars represent standard errors from at least three independent experiments. Red boxes indicate prevalent strains, while blue boxes present sporadic strains.

All IC I strains and some IC II strains formed biofilm, albeit at the varying levels, whereas a group of IC II strains that were genetically close according to the PFGE analysis (Povilonis *et al*., 2013), namely, II-d, II-dB, II-e, II-eA, II-f, II-g and II-h, showed extremely weak biofilm-forming ability or entirely lacked this phenotype (Figure 3B). Biofilm non-forming phenotype of these isolates correlated with their low surface hydrophobicity according to the SAT assay.

The majority of IC I and a part of IC II strains poorly survived desiccation, yielding only 0.005 to 1.3 percent of survived cells (Figure 3C). However, a group of hydrophilic IC II strains was highly resistant to desiccation and displayed two to fourteen times higher resistance compared with the rest of the IC II strains (Figure 3C). The decreased surface hydrophobicity possibly might increase water retention in the bacterial cell wall and thus contribute to the desiccation resistance. Moreover, we observed that the hydrophilic IC II strains II-e and II-f, displaying the highest desiccation resistance among tested strains, had approximately two-fold thicker cell wall compared with the hydrophobic strains II-a and II-b and a capsule-deficient strain II-h according to the TEM analysis (Figures 4A-F).

The specific adherence of *A. baumannii* clinical strains to the mouse lung epithelial LL/2 cells was poor (Figure 3C). The IC I strains adhered at a rate of 0.3% to 0.8%, while the adhesion of IC II strains was more pronounced and yielded 0.5% to 3.5% rate. In the IC II group of hydrophilic strains, in particular II-a2, II-c, II-d, II-eA, II-f, II-g and II-h strains showed a clear trend of increased ability to adhere to the epithelial cells (Figure 3C).

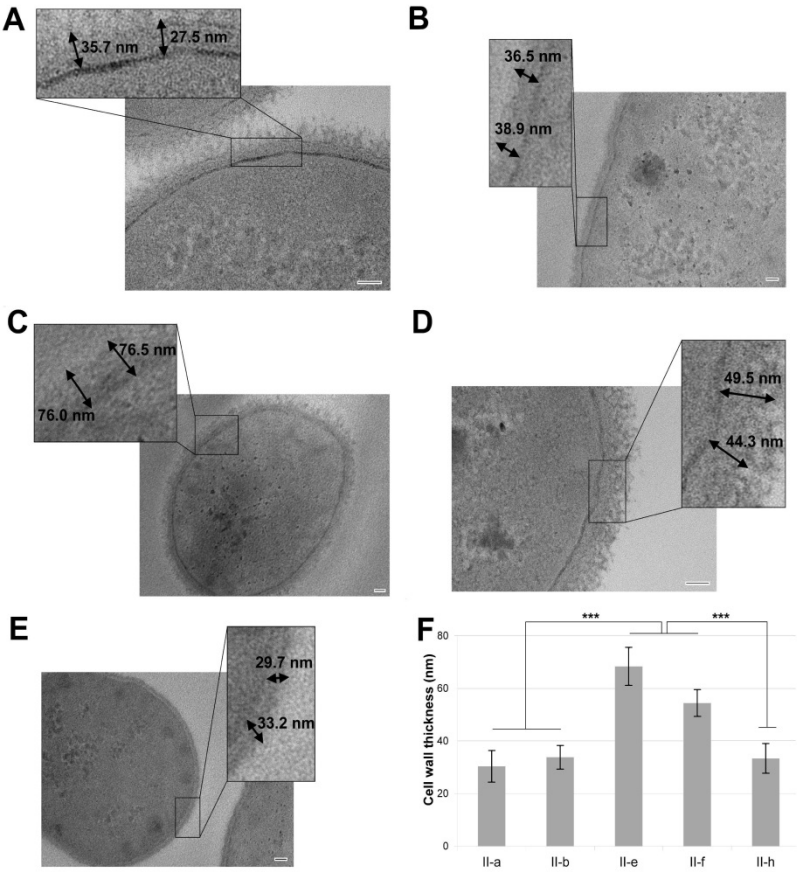


**Fig. 4.** Cell wall thickness of *A. baumannii* strains with different surface hydrophobicity, assessed by the transmission electron microscopy. *A. baumannii* II-b (A), II-a (B), II-f (C), II-e (D) and II-h (E) strains were analysed. Scale bar is 50 nm. Double arrows in the larger scale insets show the calculated thickness of the cell walls. F - average of cell wall thickness of the strains. Error bars represent standard deviations from three measurements of three different cells, significance was assessed by ANOVA, ($p<0.001$).

Whereas our study confirms a link between *A. baumannii* hydrophobicity and biofilm formation as well as adherence to the abiotic surface, it demonstrates that hydrophobic phenotype renders *A. baumannii* to become more sensitive to desiccation and weakens its ability to adhere to the epithelial cells. Taken together, these results demonstrate that a high cell surface hydrophobicity of *A. baumannii* impacts important virulence traits. The aforementioned

results imply, that IC I and IC II strains might use different cell surface-related properties for an attachment to the host cells.

It is important to note, that *A. baumannii* strains with hydrophilic nature were mostly sporadic compared to the pandemic (prevalent) strains, which largely exhibited hydrophobic cell surface characteristics. This indicates that hydrophobic cell surface is a favorable property contributing to the prevalence of *A. baumannii* in the clinical environment. While hydrophobic surface enables bacteria to adhere to the plastic surface, e.g. medical devices, hydrophilic characteristics may increase the bacterial virulence inside the host, because hydrophilic strains exhibit increased adhesion to lung epithelium cells and contain thicker capsular layer, which could contribute to the bacterial resistance to antimicrobial activity inside the host.

Our observations with IC II strains also suggest that genetically related *A. baumannii* strains belonging to the same clonal lineage, display a marked variation in the surface-related properties. Therefore, we hypothesized that these phenotypic differences could have different impact on their virulence properties and decided to investigate them more thoroughly.

Therefore, we investigated virulence of *A. baumannii* using nematodes fertility assay and mouse sepsis infection model. First, to assess the virulence in *C. elegans*, we have selected a set of IC II strains, displaying different properties of surface hydrophobicity, resistance to desiccation and ability to adhere to the lung epithelial cells. The strains II-b and II-a are hydrophobic, show poor resistance to desiccation and have a weak capacity to adhere to the epithelial cells, whereas strains II-f, II-e and II-h are hydrophilic, highly resistant to desiccation and show cell adherence ability. All selected strains except II-h, express CPS under laboratory growth conditions (Figure 2). *C. elegans* fertility assay demonstrated that a total number of progeny after three days upon infection was approximately 2 times lower in nematodes infected with the hydrophilic strains II-f, II-e and II-h compared to those infected with the hydrophobic strains II-a and

II-b and the difference was statistically significant ($p<0.01$) (Figure 5). Notably, strain II-h, being capsule-deficient under laboratory conditions, showed similar virulence features in *C. elegans* compared with capsule-producing strains II-e and II-f.

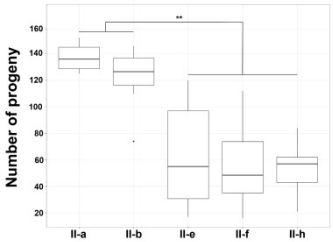


**Fig. 5.** *C. elegans* fertility assay. Box plot of the count of nematodes progeny after three days of incubation in the presence of *A. baumannii* IC II lineage strains exhibiting different cell surface hydrophobicity. Data are from three independent experiments, three to four plates were used in the each experiment. Black lines represent medians and whiskers – minimum to maximum values, significance was assessed by ANOVA ($p<0.01$).

Next, we used an experimental murine model to access the ability of selected IC II strains to establish a systemic infection. Representative IC II strain II-a with hydrophobic cell surface properties, II-f strain with hydrophilic character, and hydrophilic II-h strain, albeit displaying capsule-non-producing phenotype were used for infection. The mice survival rates were monitored for several days. The mice infected with II-f strain showed two-fold higher mortality rate compare to those infected with II-a and II-h strains (80% vs. 40%) (Figure 6A). Spleens from the mice, infected with the II-f strain and examined post-mortem had 10 times higher bacterial load compared with those from the mice infected with II-a strain, and over 30 times higher load compared with those infected with capsule-deficient II-h strain (Figure 6B). Furthermore, the higher yield of bacteria was detected in spleen from the mouse, which survived after 48 hours upon inoculation with II-f strain, while in the case of II-a strain the load of survived bacteria was mainly lower and only a few II-h colonies were observed after mice sacrifice and examination of spleens (Figure 6C).

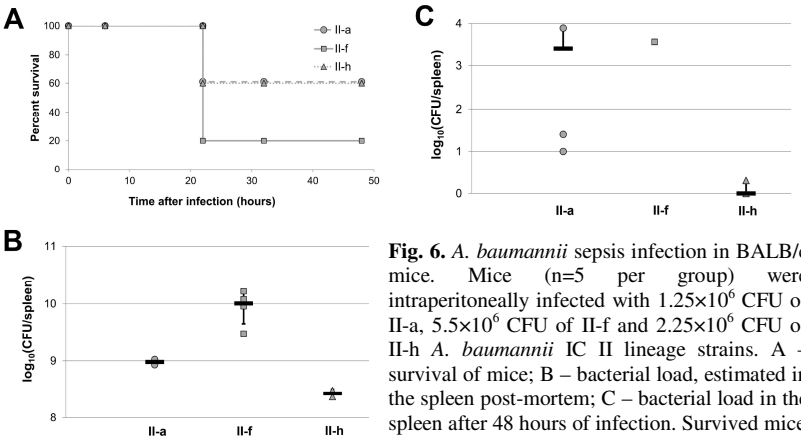


**Fig. 6.** *A. baumannii* sepsis infection in BALB/c mice. Mice (n=5 per group) were intraperitoneally infected with $1.25\times10^6$ CFU of II-a, $5.5\times10^6$ CFU of II-f and $2.25\times10^6$ CFU of II-h *A. baumannii* IC II lineage strains. A – survival of mice; B – bacterial load, estimated in the spleen post-mortem; C – bacterial load in the spleen after 48 hours of infection. Survived mice were sacrificed and spleens were tested for bacterial loads. Black lines in B and C represent an average, error bars represent standard deviations.

Therefore, the low cell surface hydrophobicity clearly impacts virulence of *A. baumannii*, although our study predicts that the capsule presence is critically needed to establish an infection in vertebrate host, but not in *C. elegans* model. This is in accordance with the observations made by Kempf *et al*. (2012) from the analysis of two *A. baumannii* strains recovered from the same patient, where a strain with hydrophobic features and biofilm forming ability did not show increased virulence compared to the strain with hydrophilic properties. Nevertheless, most of our tested *A. baumannii*, including outbreak strains formed biofilm, whereas IC II strains with hydrophilic character, increased desiccation resistance and adherence to the epithelium cells, were mostly sporadic. However, the listed features of hydrophilic *A. baumannii* strains might be superior at certain conditions such as long periods dryness or at the onset of host colonization and such strains might pose a high infection risk.

In order to understand the origin of differences in hydrophobicity, yielded by clinical *A. baumannii* strains, hydrophobic II-a and hydrophilic II-f strains were used for total DNA extraction and sequencing (Illumina, Thermo Fisher Scientific). Results

demonstrated that II-a and II-f genomes were very similar. We identified 12 single nucleotide polymorphisms (SNP) in genes, mostly related to metabolism. However, analysis of sequencing data has shown that II-f strain lacked pACICU2-type plasmid, which was present in II-a strain, while a set of other plasmids carried by both strains were the same. The PCR, targeting pACICU2-type plasmid, confirmed its presence in *A. baumannii* strains with hydrophobic cell surface properties, while all hydrophilic strains lacked this plasmid. These results indicate an association between pACICU2-type plasmid and *A. baumannii* cell surface hydrophobicity and virulence-related features.

## Characterization of virulence-related factors in *A. baumannii*

In order to identify new *A. baumannii* virulence factors and possible drug targets, next, by bioinformatic search we have identified *A. baumannii* homologues of surface-associated virulence factors, found in other bacterial pathogens. Genes, coding for selected proteins, were inactivated by markerless gene deletion method (Oh *et al*., 2013) in strain $Ab_{IC\ I}$, belonging to one of the most prevalent clonal lineages, IC I with a common sequence type ST231. Generated mutants were tested for their virulence-related features. BrkB, Sel1 and PqiB mutants showed no changes in virulence-related properties (Table 2). However, OmpA, Blp1, YgaU and GltI deletion mutants displayed reduced virulence using *in vivo* infection model. Moreover, bacterial motility was affected in OmpA and YgaU mutants. *A. baumannii*, deficient in ability to produce high molecular mass (~330 kDa) adhesin Blp1, showed reduced biofilm formation, adhesion to lung epithelium cells and virulence *in vivo*. OmpA deletion mutant demonstrated alterations in multiple virulence-related features.

OmpA is 38 kDa outer membrane protein, consisting of membranous β-barrel domain and periplasmic peptidoglycan

associated C-terminal domain (Jahangiri *et al*., 2017; Iyer *et al*., 2018). Park *et al*. (2012), by comparison of OmpA-like proteins from various human pathogens, have identified two absolutely conservative amino acids D271 and R286 in the C-terminal OmpA-like domain of *A. baumannii* OmpA. These residues have been shown to be critical for non-covalent association of OmpA to diaminopimelate amino acid, a component of *A. baumannii* peptidoglycan, as demonstrated by the isothermal titration calorimetry using purified recombinant OmpA proteins with D271A

**Table 2.** The impact of gene coding for putative virulence factors on *A. baumannii* phenotype. + effect observed, - no effect observed.

| ***A. baumannii* homologue** | **Virukence factor in bacterial pathogen and its function** | ***A. baumannii* phenotypes** | | | | | |
|---|---|---|---|---|---|---|---|
| | | Motility | Biofilm | Resistance to serum | Adhesion to cells | *C. elegans* infection | Balb/c mice infection |
| **OmpA** | *E. coli* OmpA; Resistance to serum and phagocytosis (Mittal *et al*., 2011) | + | + | + | + | + | + |
| **Blp1** | *S. typhimurium* BapA; Biofilm (Latasa *et al*., 2005) | - | + | - | + | + | + |
| **YgaU** | *E. coli* YgaU; Stress response (Bernal-Cabas *et al*., 2015) | + | - | - | - | - | + |
| **GltI** | *C. jejuni* PebA; Adhesion to cells (Leon-Kempis *et al*., 2006) | - | - | - | - | - | + |
| **BrkB** | *B. pertussis* BrkB; Resistance to serum (Shrivastava *et al*., 2009) | - | - | - | - | - | - |
| **Sel1** | *L. pneumophila* LpnE; Resistance to phagocytosis (Newton *et al*., 2007) | - | - | - | - | - | - |
| **PqiB** | *V. cholerae* VP1611; Adhesion to cells (Krachler and Orth, 2011) | - | - | - | - | - | - |

and R286A substitutions, respectively (Park *et al*., 2012). Therefore, we decided to investigate the role of association of OmpA protein to peptidoglycan on the virulence characteristics of *A. baumannii* clinical strain $Ab_{IC\ I}$. We have examined wild type strain its Δ*ompA* mutant and $ompA_{D268A}$ complemented strain, carrying substitution of one of the key residues (D271 corresponds D268 in OmpA variant from $Ab_{IC\ I}$ strain), required for OmpA interaction with the peptidoglycan.

We analyzed the biofilm forming capacity of $Ab_{IC\ I}$ and $Ab_{IC\ I}$Δ*ompA* mutant, complemented with a control plasmid and with plasmids p*ompA* or p$ompA_{D268A}$. The strains were tested for their initial attachment to the plastic by incubating in LB medium at 37°C for 2 hours. The biofilm analysis was undertaken by confocal laser scanning microscopy (CLSM). As can be seen in Figures 7A-B, two hours after seeding, most of the $Ab_{IC\ I}$ cells, attached to the plastic were viable, as judged from the dominance of SYTO9 stained cells (green color). The *ompA* gene knockout in $Ab_{IC\ I}$ resulted in approximately 65% increase in the amount of propidium iodide (PI; red color) stained cells, which in addition tend to form prolonged bacterial chains, most likely due to the impairment of cell division, as this was demonstrated in other bacteria with impaired peptidoglycan maintenance (Arrigucci and Pozzi, 2017; Pazos *et al*., 2018). The $Ab_{IC\ I}$Δ*ompA* complementation with p*ompA* plasmid resulted in a significantly lowered number of PI-stained cells and an absence of prolonged bacterial cells chains (Figure 7A), whereas in the $Ab_{IC\ I}$Δ*ompA* cells with control plasmid this phenotype was clearly visible (Figure 7B). The introduction of plasmid-borne *ompA* allele with D268A substitution into $Ab_{IC\ I}$Δ*ompA* strain efficiently reduced the number of PI-stained cells, although was not able to eliminate the phenotype of prolonged cell chains (Figure 7B).

After 24 hours of incubation *A. baumannii* mature biofilm structures were examined (Figure 7C). The PI-stained cells were found to be distributed mostly on the top of biofilm formed by $Ab_{IC\ I}$ strain. In contrast, the biofilm of $Ab_{IC\ I}$Δ*ompA* mutant contained substantial amount of PI-stained cells and prolonged cell chains were also evident. Both of these phenotypes were largely eliminated by the introduction of p*ompA* plasmid. Similarly, the $Ab_{IC\ I}$Δ*ompA* strain complementation with the plasmid carrying *ompA*D268A allele also resulted in reduced amount of PI-stained cells in a mature biofilm, however, did not eliminate the phenotype of prolonged cell chains (Figure 7C).

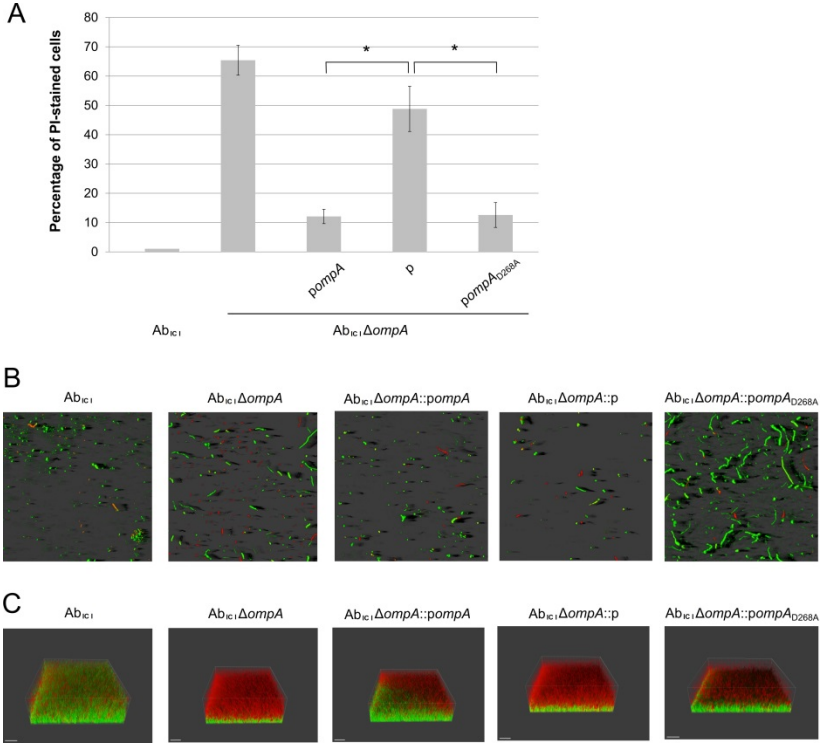


**Fig. 7.** CLSM analysis of *A. baumannii* biofilm formation. A – number of propidium iodide-stained bacteria after 2 hours of incubation, compared to the total amount of cells expressed as a percentage; error bars represent standard deviations from six measurements of six different CLSM pictures, significance was assessed by t-test, (*P<0.05). B – visualization of initial attachment to the plastic of *A. baumannii* strains, assessed after 2 hours of incubation; bacteria were stained with SYTO9 (green) and propidium iodide (red). C – mature biofilm formation after 24 hours of incubation.

It has been previously shown that *A. baumannii* OmpA is required for bacterial attachment to the epithelial cells (Gaddy *et al*., 2009). Therefore, we were interested, whether C-terminal domain of OmpA plays any role in the expression of this virulence trait. For this purpose, the $Ab_{IC\ I}$ strain and its Δ*ompA* mutant complemented either with an empty plasmid or with plasmid carrying the *ompA*D268A allele were tested for the ability to adhere to the mice lung epithelium cells LL/2. As can be seen in Figure 8A, the Δ*ompA* mutant showed approximately 6-fold decrease in adhesion compared to the parent

strain thereby confirming the role of OmpA in supporting the adhesive properties of *A. baumannii*. The *ompA* allele restored the phenotype of Δ*ompA* mutant, although not fully. Notably, the *ompA*D268A allele was nearly deficient in complementation ability being comparable to that of empty plasmid (Figure 8A).

The ability to avoid host defense systems such as complement is a crucial feature in establishing the infection by *A. baumannii* (Russo *et al*., 2010). OmpA protein is viewed as one of the most important virulence factors involved in mediating *A. baumannii* resistance to human serum components, since OmpA ability to bind and inactivate complement factor H has been demonstrated (Kim *et al*., 2009). Therefore, we tested the capability of $Ab_{IC\ I}$ strain and its Δ*ompA* mutant complemented with *ompA*D268A allele and with the control plasmid to avoid serum-mediated killing. For this purpose bacteria

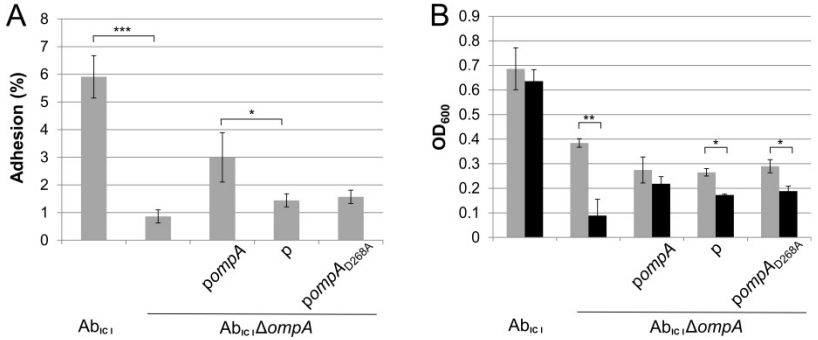


**Fig. 8.** *A. baumannii* adhesion to LL/2 cells and resistance to serum. A – bacterial adhesion to the LL/2 cells was expressed as a percentage of the CFUs of adhered bacteria compared to the total number of CFUs of the initial inoculum; error bars represent standard errors from at least three independent experiments. B – effect of *ompA* gene to resistance to serum-mediated killing in *A. baumannii* strains; strains were grown in LB media supplemented with 80% of heat-inactivated (grey bars) or active (black bars) FBS for 13 hours and $OD_{600}$ was measured; error bars represent standard deviations of three independent experiments. Significance was assessed by t-test (***P<0.001; **P<0.01; *P<0.05).

were grown in LB media supplemented with 80% of active or heat-inactivated FBS. Δ*ompA* mutant exhibited significantly reduced growth in active serum-supplemented media compared to that with the heat-inactivated serum (Figure 8B). The complementation with *ompA* restored serum resistance, whereas the presence of

*ompA*D268A variant was not able to eliminate serum sensitivity of Δ*ompA* strain, indicating that OmpA protein interaction to peptidoglycan contributes to *A. baumannii* resistance to serum complement components.

For the validation of the effect of D268A substitution in OmpA on *A. baumannii* virulence *in vivo* we have accessed nematodes fertility by counting worm progeny after three days upon *A. baumannii* infection. We have observed that *ompA* deletion impaired virulence of $Ab_{IC\ I}$ strain, which was fully complemented by the *ompA* gene supplied *in trans* (Figure 9). However, the *ompA* allele with D268A substitution did not rescue the phenotype indicating the importance of association of OmpA to peptidoglycan on *A. baumannii* infection *in vivo*.

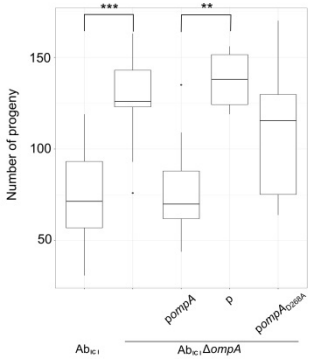


**Fig. 9.** *A. baumannii* virulence in nematode infection model. *C. elegans* fertility was evaluated after 3 days of nematodes growth in the presence of *A. baumannii* bacteria; box plot represents the count of nematodes progeny after incubation; data were obtained from three independent experiments, three plates were used in the each experiment; black lines represent medians and whiskers – minimum to maximum values; significance was assessed by ANOVA (***P<0.001; **P<0.01).

## Blp1 protein as vaccine candidate

One of the most promising strategies against MDR *A. baumannii* infections is a development of vaccines (Ahmad *et al*., 2016; Dickey *et al*., 2017). The surface or capsular polysaccharides were researched as potent immunization agents (Russo *et al*., 2013), however, their high diversity of polysaccharides (Hu *et al*., 2013) among *A. baumannii* clinical strains limits development of universal vaccine. The inactivated whole *A. baumannii* cells and outer membrane vesicles (OMVs) also demonstrated the induction of

immune-response using murine models (Huang *et al*., 2014; KuoLee *et al*., 2014). Compared to the complex antigen mixtures, the pure proteins as vaccine antigens are more desirable due to the safety issues. Indeed, several *A. baumannii* outer membrane proteins have been used for vaccine research, mostly the abundant outer membrane proteins (Zhang *et al*., 2016; Singh *et al*., 2016). However, the vast majority of clinical *A. baumannii* strains possess a thick outer layer of polysaccharides (capsule), which efficiently protects pathogens from the host immunity (Russo *et al*., 2010; Skerniškytė *et al*., 2019) by shielding antigens, present on the cell surface of the pathogen. Therefore, the surface-exposed proteins, penetrating the capsule layer, might show superiority as candidates for vaccine development (Badmasti *et al*., 2015).

Our observation that *A. baumannii* Blp1 proteins, encoded by *blp1* gene variants, share conservative C-terminal 160 amino acid fragment, allowed proposing it as a suitable antigen candidate for investigation of immune-stimulatory properties of a long (~330 kDa) *A. baumannii* adhesin Blp1. To increase the number of antigenic moieties displayed at the likely surface-exposed C-terminus of Blp1, 712 amino acids of C-terminal Blp1 from $Ab_{IC\ I}$ (residues 2652-3363) with a N-terminus His-Tag was purified by affinity chromatography as described in Materials and Methods section. As a control purified OmpA protein was used, since its immunogenic properties was described earlier in mice infection model (Lin *et al*., 2013).

First, the cytotoxicity of purified recombinant Blp1 fragment and OmpA protein has been evaluated using mouse lung epithelium LL/2 cells. The purified antigens demonstrated a mild suppressing effect on the growth of LL/2 cells *in vitro* in the concentration range from 2.5 to 10 μg/ml of Blp1 and OmpA allowing the maintenance of approximately 90%– 83% and 95%– 91% viability of the cells for 24 hours, respectively. Mild cytotoxicity of purified antigens towards lung epithelium cells suggests that they represent safe candidate antigens for vaccination.

For the active immunization, the 2 μg of the recombinant Blp1 fragment and OmpA protein were injected into BALB/c mice (n=5 per group) intramuscularly at the frequency of every two weeks, three times in total. At the fourth day after the last immunization, the blood samples were taken and obtained antisera were used for the determination of Blp1 or OmpA-specific-IgG titer by ELISA. The obtained results showed the induction of specific IgG response in the animal group immunized with antigens compared to the control group (Figures 10A-B). Then, at the day 42nd, the intraperitoneal challenge with *A. baumannii* $Ab_{IC\ I}$ strain ($10^8$ CFU per mouse) has been undertaken and the mice survival rates were monitored for seven days. The animal group, which received Blp1 specific antigen, demonstrated the 60% survival rate, OmpA-immunized group demonstrated 40% survival rate, whereas no survival in the control group was recorded (Figure 10C). Since Blp1 fragment demonstrated better survival chances, it was used for further investigation.

For the passive immunization, the antisera, obtained from the mice immunized with Blp1 specific antigen and from the control group, treated with PBS, were used. Antisera were injected intraperitoneally into the naïve mice (n=3 per group). Six hours later, animals were challenged with *A. baumannii* by inoculating $5 \times 10^7$ CFUs of $Ab_{IC\ I}$ strain. The survival was monitored for 45 days. As can be seen in the results presented in Figure 10D, a group, which received antiserum against Blp1 specific antigen, yielded 100% survival rate, whereas group of mice, that received serum from the control group, resulted in 0% survival indicating an efficient protective immunity to *A. baumannii* infection, exerted by Blp1 specific antiserum.

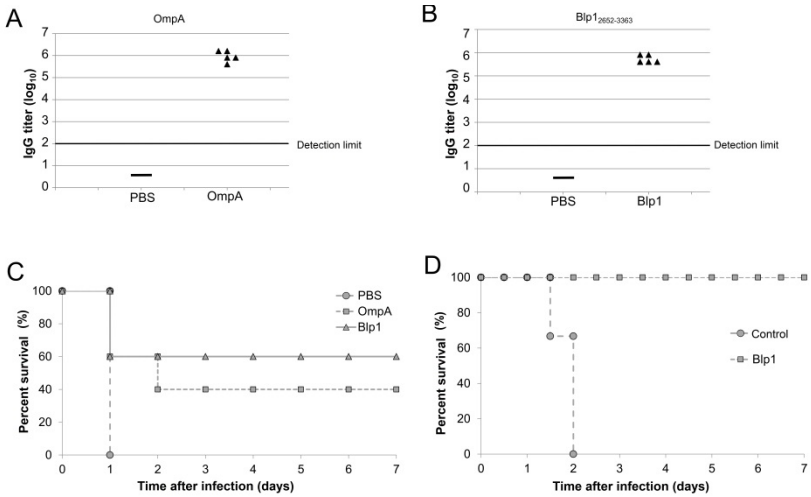


**Fig. 10.** The effects of OmpA protein and Blp1 C-terminal fragment as an immunization agent. Titers of OmpA (A) and Blp1(B) -specific IgG in mice were detected by ELISA (n=5 mice per group); naïve sera obtained from mice, that received adjuvant with PBS were used as a control; horizontal line represents detection limit of the assay. C – mice survival rates after active vaccination using *A. baumannii* sepsis model; mice (n=5 mice per group) were challenged with $10^8$ CFUs of $Ab_{IC\ I}$ and were monitored for seven days; D – passive vaccination effect on the mice survival using *A. baumannii* sepsis model; control group received the serum obtained from mice, immunized with PBS and Freund's adjuvant; mice (n=3 mice per group) were challenged with $5 \times 10^7$ CFU of $Ab_{IC\ I}$ and were monitored for 45 days.

To identify the main mechanism responsible for antimicrobial activity of obtained Blp1-antiserum, the *A. baumannii* $Ab_{IC\ I}$ (international clone I, ST231) and $Ab_{IC\ II}$ (international clone II, ST208) strains were incubated for one hour with heat-treated (to inactivate complement components) Blp1-specific and naïve serum in the presence or absence of J744 macrophages. The presence of macrophages increased killing against both $Ab_{IC\ I}$ and $Ab_{IC\ II}$ strains by approximately 20%, when inactivated Blp1-antiserum, but not naïve serum was present (Figure 11). In the absence of macrophages, killing efficiency of *A. baumannii* was negligible regardless the origin of inactivated antisera. Therefore, an increase of opsonophagocytic killing of *A. baumannii* using heat-inactivated Blp1-antiserum indicates its macrophage-mediated, but not complement-depended, antimicrobial activity.

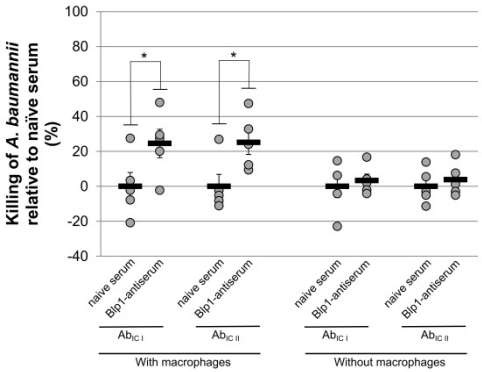


**Fig. 11.** Blp1-antiserum-depended opsonophagocytic killing of *A. baumannii* IC I and IC II strains. The bactericidal killing activities of Blp1-antiserum and naïve serum were analyzed on *A. baumannii* $Ab_{IC\ I}$ and $Ab_{IC\ II}$ strains in the presence and absence of J774 macrophages; each dot represents one mouse, black lines indicate medians and whiskers represent standard errors; significance was assessed by t-test, (*P<0.05).

In conclusion, using both active and passive immunization approaches we demonstrated, that Blp1-specific antigen is a suitable vaccine candidate against *A. baumannii* infection. The Blp1-antiserum induced an opsonophagocytic killing of two common *A. baumannii* strains ST231 (IC I) and ST208 (IC II), indicating the stimulation of an effective immune response against clonally disseminated MDR *A. baumannii*.

## CONCLUSIONS

1. Pandemic *A. baumannii* strains have different virulent properties;
2. Cell surface hydrophilicity is associated with the increased *A. baumannii* virulence, but is not a favorable property for the spread of clinical strains;
3. *A. baumannii* OmpA, Blp1, YgaU and GltI proteins are virulence factors *ex vivo* and *in vivo*;
4. The interaction of OmpA periplasmic domain with peptidoglycan determines the multifunctional role of the protein in *A. baumannii* pathogenesis;
5. C-terminal fragment of *A. baumannii* Blp1 protein is an effective antigen for active immunization, while the Blp1-antiserum protects against *A. baumannii* infection when passive immunization is applied.

## List of publications

**Skerniškytė J**, Karazijaitė E, Deschamps J, Krasauskas R, Briandet R Sužiedėlienė E. (2019). The Mutation of Conservative Asp268 Residue in the Peptidoglycan-Associated Domain of the OmpA Protein Affects Multiple *Acinetobacter baumannii* Virulence Characteristics. Molecules. 24:1972;

**Skerniškytė J**, Krasauskas R, Péchoux C, Kulakauskas S, Armalytė J, Sužiedėlienė E. (2019). Surface-Related Features and Virulence Among *Acinetobacter baumannii* Clinical Isolates Belonging to International Clones I and II. Front Microbiol. 9:3116.

## Conference presentations

Poster presentation: **Skerniškytė J**, Krasauskas R, Sužiedėlienė E. "Characterization of Blp1 adhesin as vaccine candidate against multidrug-resistant *Acinetobacter baumannii*", 12th Symposium on the Biology of Acinetobacter (Acinetobacter2019), 4-6 September, 2019, Frankfurt, Germany;

Poster presentation: Karazijaitė E, **Skerniškytė J**, Sužiedėlienė E. "Isolation and Characterization of Outer Membrane Vesicles from Opportunistic Pathogen *Acinetobacter baumannii*", The Coins 2019, 26-28 February, 2019, Vilnius, Lithuania;

Oral and poster presentation: **Skerniškytė J**, Sužiedėlienė E. "Blp1 protein as new vaccine candidate against infections caused by multidrug-resistant *Acinetobacter baumannii*". 26$^{th}$ Young Research Fellows Meeting (French Medical Chemistry Society), 20-22 February, 2019, Paris, France;

Oral presentation: **Skerniškytė J**, Karazijaitė E, Krasauskas R, Armalytė J, Sužiedėlienė E. "Potencialių virulentinių genų svarba klinikinių *Acinetobacter baumannii* padermių patogenezei", 10th Young Scientists Conference "Bioateitis: gamtos ir gyvybės mokslų perspektyvos", 07 December, 2017, Vilnius, Lithuania;

Poster presentation: **Skerniškytė J**, Karazijaitė E, Deschamps J, Briandet R, Péchoux C, Kulakauskas S, Krasauskas R, Armalytė J, Sužiedėlienė E. "The impact of putative virulence genes on pathogenicity displayed by clinical *Acinetobacter baumannii* strains", 7th Congress of European Microbiologists (FEMS 2017), 9-13 July, 2017, Valencia, Spain;

Poster presentation: **Skerniškytė J**, Krasauskas K, Armalytė J, Sužiedėlienė E. "Distribution of putative virulence factors in *Acinetobacter baumannii* clinical strains isolated from Lithuanian hospitals and their impact on pathogenesis", XIV International Conference of the Lithuanian Biochemical Society, June 28-30, 2016, Druskininkai, Lithuania.

## Financial support

Part of the research presented in the dissertation was financed by the Research Council of Lithuania under the project 09.3.3.-LMT-K-712-10-0095.
Ersamus+ has funded a 3-month internship at the Micalis Institute in France.
Federation of European Microbiological Societies (FEMS) has partly funded participation in: *the 7th Congress of European Microbiologists (FEMS 2017*) and *the 12th Symposium on the biology of Acinetobacter (Acinetobacter2019).*

# SANTRAUKA

Šiuo metu ligoninėse plinta daugiavaisčiu atsparumu pasižymintys *A. baumannii* izoliatai, kurių sukeltas infekcijas išgydyti yra labai sunku dėl naudojamų antibiotikų neveiksnumo. Šio tyrimo metu apibūdinome klinikinius *A. baumannii* izoliatus pagal įvairius fenotipus, susijusius su bakterijų virulentiškumu. Nustatėme, kad izoliatai, priklausantys skirtingiems pandeminiams klonams, skiriasi savo judėjimo, pelikulės formavimo bei sintetinamos kapsulės egzopolisacharidų profiliais. Parodėme, kad bakterijos paviršiaus hidrofobiškumas nulemia daugelį su virulentiškumu susijusių *A. baumannii* savybių, o gebėjimas adhezuotis prie plastiko dėl ląstelės paviršiaus hidrofobiškumo yra vyraujanti pandeminių izoliatų savybė. Antrojoje darbo dalyje apibūdinome *A. baumannii* paviršiaus baltymus OmpA, Blp1, YgaU ir GltI kaip svarbius virulentiškumo veiksnius tiek *ex vivo*, tiek *in vivo*. Parodėme, kad membraninio baltymo OmpA sąveikos su peptidoglikanu praradimas lemia *A. baumannii* virulentiškumo sumažėjimą. Taip pat abipūdinome Blp1 baltymo C-galinį fragmentą kaip naują vakcinos kandidatą prieš klinikinės kilmės *A. baumannii* infekcijas taikant tiek aktyvią, tiek pasyvią vakcinacijas pelių infekcijos modelyje.

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**List of publications**


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## ACKNOWLEDGEMENTS

I am grateful to my supervisor Prof. Dr. Edita Sužiedėlienė for the valuable discussions and remarks, patience and sincere concern as well as all help with this research.

I thank my colleagues Dr. Julija Armalytė and Renatas Krasauskas for the practical advices and inspiring discussions. Many thanks to Renatas Krasauskas for his help in analyzing genome sequencing results, also help with bacterial movement and pellicle formation experiments and at various other moments of laboratory life. I am also grateful to Emilija Karazijaitė, Gabija Lauciūtė and Gabija Goptaitytė for their help.

I thank the whole team of the Department of Biochemistry and Molecular Biology for their invaluable support.

I sincerely thank Prof. Dr. Nomeda Kuisienė and Dr. Milda Plečkaitytė for the careful reading of the thesis, valuable insights and comments.

I want to thank Prof. Dr. Kęstutis Sužiedėlis, Prof. Dr. Aurelija Žvirblienė and Dr. Aušra Sasnauskienė for the consultations on eukaryotic cell cultivation. I am grateful to Dr. Martina Rudgalvytė for the advices on nematode cultivation. Thank you very much to Dr. Virginija Bukelskienė and Ieva Rinkūnaitė for the consultations and help on the experiments with mice. Thanks to Prof. Dr. Rolandas Meškys for the *E. coli* strain. Thank you to Dr. Audrius Gegeckas for *Bacillus* spp. strain. I would like to thank the Department of Eukaryote Gene Engineering at the Institute of Biotechnology and Dr. Justas Lazutka for the ability to use transmission electron microscopy. I thank to Proteomics Center (VU Institute of Biochemistry) for proteomic analysis.

I sincerely thank Thermo Fisher Scientific for the sequencing of *A. baumannii* genomes.

I would like to thank the wonderful team of Micalis Institute, especially dr. Saulius Kulakauskas for all help.

Thank you very much to Milda Zilnytė and Indrė Dalgėdienė for their invaluable discussions.

Finally, I would like to thank my whole family for all their care and special thanks to Vytautas Petkevičius, whose permanent support provided the strength through this entire journey.

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Vilnius University press
Saulėtekio al. 9, LT-10222 Vilnius
Email: info@leidykla.vu.lt,
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Edition 15 copies