Cloning of *ompA* gene from *Acinetobacter baumannii* into the eukaryotic expression vector *pBudCE4.1* as DNA vaccine

Hossein Ansari^{1,5} · Abbas Doosti² · Mohammad Kargar³ · Mahdi Bijanzadeh⁴ ·
Mojtaba Jaafarinia⁵

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Abstract Antibiotic resistant features of *Acinetobacter baumannii* is partly due to the decreased outer membrane proteins (OMPs) permeability. The OmpA is one of the most conserved proteins among *A. baumannii* with a considerable antigenic potential to stimulate the multidimensional immune system responses. The present study was aimed to clone the *ompA* gene into the eukaryotic expression vector with potential as DNA vaccine. The *ompA* gene of *A. baumannii* was amplified using polymerase chain reaction (PCR). The target DNA was cloned and sub-cloned into the pTZ57R/T and *pBudCE4.1*

vectors, respectively. The recombinant vectors containing *ompA* were then validated using colony PCR, vector sequencing and double-digestion strategies. The *pBudCE4.1-ompA* recombinant plasmid was transfected into the human dermal fibroblast cells (HDF) and presence of *ompA* transcript and protein was evaluated using reverse transcribed-PCR (RT-PCR) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Our finding from colony PCR, sequencing and enzyme double digestion result confirmed that target gene has been successfully inserted into the pTZ57RT and *pBudCE4.1*. The presence of an expected band (1112 bp) in RT-PCR as well as a ~ 38 kDa band during SDS-PAGE showed that the recombinant *pBudCE4.1-ompA* construct was efficiently transfected into the HDF cells and expressed. Altogether, our observation demonstrated that the recombinant *pBudCE4.1-ompA* construct was successfully produced although further experiments are needed.

✉ Abbas Doosti
Abbasdoosti@yahoo.com

Hossein Ansari
hosseinansari62@gmail.com

Mohammad Kargar
microkargar@gmail.com

Mahdi Bijanzadeh
mbijanz@yahoo.com

Mojtaba Jaafarinia
jaafarinia@yahoo.com

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Abbreviations

<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
cDNA	Complementary DNA
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
g	Gram
GMSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
HDF	Human dermal fibroblast
IDSA	Infectious Diseases Society of America

- ¹ Department of Molecular Genetics, Fars Science and Research Branch, Islamic Azad University, Shiraz, Islamic Republic of Iran
- ² Biotechnology Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Islamic Republic of Iran
- ³ Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Islamic Republic of Iran
- ⁴ Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Islamic Republic of Iran
- ⁵ Department of Molecular Genetics, Marvdasht Branch, Islamic Azad University, Marvdasht, Islamic Republic of Iran

IPTG	Isopropyl- β -D-thiogalactoside
LB	Luria-Bertani
ml	Milliliter
min	Minute
MDR	Multidrug resistance
NTC	Negative control
OMP	Outer membrane protein
OmpA	Outer membrane protein A
OMVs	Outer membrane vesicles
PCR	Polymerase chain reaction
PNAG	Polysaccharide poly- <i>N</i> -acetyl- β -(1-6)-glucosamine
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
U	Unit

Introduction

Acinetobacter baumannii is one the most cause of endemic and epidemic nosocomial infections including bacteremia, urinary tract infection, wound infections and meningitis [1]. The resistant feature of the bacteria to the broad spectrum of antibacterial agents such as penicillins, carbapenems, aminoglycosides and cephalosporins makes them untreatable and increase the pathogen-associated mortality rate unbelievably [2, 3]. In the last decade, the multidrug resist strains of *A. baumannii* are rapidly emerged in a way that Infectious Diseases Society of America (IDSA) introduces the *A. baumannii* as one the most dangerous microbes [4]. Therefore, the need to develop an efficient vaccination system against this opportunistic human pathogen seems urgent [5]. Several molecular mechanisms seems to be involved in multidrug resistance (MDR) phenotype of *A. baumannii* among which decreased outer membrane protein (OMP) permeability, overexpression of efflux pumps are relatively well studied [6–8]. The OMPs are actually bacterial porins seems to be associated with multi drug resistant phenotype of *acinetobacter* species and act as cell surface channels to export the antibacterial agents outside the cell [9]. The outer membrane protein A (OmpA), is one the most abundant porins in gram negative bacteria which can regulate the cellular permeability and *A. baumannii* is no exception [10]. The OmpA attaches to eukaryotic cells, translocate into the nucleus and induce cell death [11]. It can also inactive the complement system's response by

capturing the serum factor H [12]. These issues trigger the idea that OMPs can be considered as potent targets to fight *Acinetobacter* species. Regarding to above mentioned information, this study was aimed to develop a DNA vaccine construct in which *ompA* gene was considered as immunogenic inducer. It is necessary to mentioned that OmpA is one of the most conserved porins in *A. baumannii* genus which is actively involved in bacterial survival, virulence, cell adhesion, host invasion and immune evasion [9, 13].

Materials and Methods

Bacterial Strains and Cell Lines

Acinetobacter baumannii ATCC 19606 and *E. coli* Nova-Blue were obtained from the Iranian Biological Resource Center (IBRC) and Cellular and Molecular Research Center of Shahrekord, University of Medical Sciences, respectively. These strains were grown under optimal conditions in Luria-Bertani (LB) agar (Merck Co., Germany) containing 5 g l⁻¹ sodium chloride, 5 g l⁻¹ yeast extract, 10 g l⁻¹ tryptone, typically combined with 15 g l⁻¹ agar (Difco, USA) overnight at 37 °C. Human dermal fibroblast cells (HDF) were purchased from the National Cell Bank of Iran, Pasteur Institute and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μ g ml⁻¹ inactivated fetal calf serum (FCS) (Gibco, USA) and 100 U ml⁻¹ penicillin, and 100 mg ml⁻¹ streptomycin (Life Technologies). All cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. The cells were trypsinized every 2 days using 0.25 mM Trypsine-EDTA (Gibco, USA).

Vectors and Plasmids

The pTZ57RT easy vector (Thermo Scientific, Lithuania) and pBudCE4.1 (Fig. 1) (Invitrogen, USA) were used as cloning and expression vectors, respectively.

Extraction of Genomic DNA from *Acinetobacter baumannii*

Genomic DNA was isolated from bacterial colonies using DNA extraction kit (Qiagen DNA Mini Kit,) according to manufacturer's instruction. The quality and quantity of final extracted DNA were checked on 1% agarose gel electrophoresis stained with ethidium bromide and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at a wavelength of 230, 260 and 280 nm.

Fig. 1 Schematic representation of recombinant pBudCE4.1 expression vector. The *ompA* gene inserted into the plasmid cloning sites using *XbaI* and *BamHI* digestion

Polymerase Chain Reaction (PCR)

The *ompA* nucleotide sequence from *A. baumannii* (Accession number: AY485227) was recruited from National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov>). Specific primers were designed using Gene Runner software version 3.5 to amplify a 1112 bp fragment from *ompA* gene, as given in Table 1. Two restriction sites for *XbaI* and *BamHI* were included in forward and reverse primers, respectively. PCR reaction was performed in a total reaction volume of 25 μ l containing 2.5 μ l of 10 $\times$ PCR buffer, 2 mM $MgCl_2$, 100 mM dNTPs, 1 μ M of each primer, 1 μ g of DNA sample and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). The final volume was adjusted with nuclease-free water. PCR temperature was optimized as initial denaturation at 95 $^{\circ}C$ for 5 min, followed by 35 cycles; denaturation at 94 $^{\circ}C$ for 1 min, annealing at 61 $^{\circ}C$ for 1 min and extension at 72 $^{\circ}C$ for 1 min. Ultimately, a final extension phase was programmed at 72 $^{\circ}C$ for 10 min. The PCR products were then evaluated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining on UV transilluminator.

Table 1 Primers for *ompA* PCR-amplification

Primer name	Oligonucleotide sequence	Amplicon size
<i>ompA-F (XbaI)</i>	5'-AGGTCTAGAATGAAATTGAGTCGTATTGC-3'	1112 bp
<i>ompA-R (BamHI)</i>	5'-CGTGGATCCTTTTACTGTTCAAGAACTC-3'	

Construction of *ompA*-Containing Recombinant Plasmids

The amplified product was purified from the gel using *AccuPrep*® Gel Purification (Bioneer, Korea) according to the manufacturer guideline and cloned into the pTZ57R/T cloning vector (Thermo Scientific, Lithuania) following the manufacturer's instruction. The *E. coli* *NovaBlue* competent cells were generated based on the calcium chloride method [14]. The competent cells were then cultured in LB agar media containing Xgal (20 mg ml⁻¹), IPTG (isopropyl- β -D-thiogalactoside) (0.1 M) and Ampicillin (100 μ g ml⁻¹) to screen the recombinant vectors at 37 $^{\circ}C$ overnight. The white colonies were selected and were cultured again in LB broth media enriched with 100 μ g ml⁻¹ Ampicillin. The recombinant vector was purified using Plasmid Mini Extraction Kit (Bioneer, Korea) as described in its protocol. To confirm the accuracy of cloning, the recombinant vector was digested with *XbaI* and *BamHI* restriction enzymes as well as PCR analysis by means of *ompA*-specific primers.

Sub-cloning of *ompA* Gene into pBudCE4.1 Eukaryotic Expression Vector

To construct the pBudCE4.1-*ompA* (Fig. 1), the pTZ57RT-*ompA* recombinant vector was double digested with *XbaI* and *BamHI*. The *ompA* was then purified using *AccuPrep*® Gel Purification (Bioneer, Korea) and gently mix with linearized pBudCE4.1 according to the instruction. T4 ligase enzyme was used to generate the pBudCE4.1-*ompA* recombinant construct. The *E. coli* *NovaBlue* competent cells were then transformed with pBudCE4.1-*ompA* and cultured in LB-Zeocine (25 μ g ml⁻¹) plates. Polymerase chain reaction and *XbaI/BamHI* double digestion were applied to verify the pBudCE4.1-*ompA* construct.

Transfection of HDF Cells with pBudCE4.1-*ompA* Construct

Transfection assay was performed using Lipofectamine 2000 reagent (Invitrogen, USA) when the confluency of HDF cells reached to 80–85%. One day before transfection, the HDF cells trypsinized and were plated in 6-well plates (1 $\times$ 10⁶ cells per well). Lipofectamin 2000 reagent and recombinant pBudCE4.1-*ompA* were diluted

by Opti-MEM separately. Diluted vector was added to the diluted lipofectamin in 1:1 ratio and incubated for 5 min at room temperature. The lipid-DNA complex was then added to the HDF cells in a serum-free DMEM for 6 h. The media was then replaced with complete fresh media containing $50 \mu\text{g ml}^{-1}$ Zeocine as a selectable marker. The HDF cells transfected with pBudCE4.1 (without gene of interest) were also considered as control of transfection (Blank).

RNA Extraction

To reveal if the transfection worked, total RNA was extracted from HDF cells (1×10^5 cells) using RNX-Plus reagent according to the manufacturer's protocol. The integrity of extracted RNA was checked on 2% agarose gel electrophoresis and the presence of 18sRNA (800 bp) and 28sRNA (1500 bp) was traced in extracted RNA samples. Additionally, the concentration and purity of the genomic DNAs were then evaluated by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) reading at wave length 230, 260 and 280 nm. The RNA was considered pure if the absorbance ratio of 260/280 nm and 260/230 nm was around 2.

Complementary DNA (cDNA) Synthesis and RT-PCR

Complementary DNA (cDNA) was synthesized using a reaction containing $500 \text{ ng } \mu\text{l}^{-1}$ RNA, 100 mM dNTPs, $10 \mu\text{l}$ $5\times$ RT-PCR buffer, 200 mM oligdT primer and $1 \text{ U } \mu\text{l}^{-1}$ M-MuLV reverse transcriptase, (Invitrogen, USA) in a total volume $20 \mu\text{l}$. The mixture was incubated at 42°C for 1 h followed by enzyme inactivation; 15 min in 70°C . The *ompA* transcript was amplified using the specific primers as shown in Table 1. The reaction was carried out in total volume of $20 \mu\text{l}$ containing 50 mM MgCl_2 , 0.25 mM of each dNTP, 2 pmol l^{-1} of each primer, $10 \text{ ng } \mu\text{l}^{-1}$ cDNA templates and 1 U of Taq DNA polymerase (Fermentas, Germany). The final volume was adjusted with nuclease-free water. PCR was optimized in thermocycler and comprised of 5 min denaturation at 95°C for initial denaturation followed by 25 cycles of denaturation; 94°C for 1 min, annealing; 61°C for 1 min, extension; 72°C for 45 s, final extension; 72°C for 2 min. A sample without DNA template (negative control: NTC) was included in all PCR reactions as negative control. The PCR products were then analyzed on 1% agarose gel electrophoresis dyed with 2% ethidium bromide and product bands were visualized under ultraviolet light.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) Analysis

Following 48 h post-transfection, the cells were harvested and centrifuged at 6000 rpm for 20 min at 4°C . Cell pallet was ultrasonically broken (300 V , $5 \text{ s} \times 3$). The obtained cell lysate was electrophoresed on SDS-PAGE to detect the recombinant *OmpA* protein based on its molecular weight. A protein lysate from HDF cells transfected with empty pBudCE4.1 vector, was also considered as blank of SDS PAGE.

Results

The Recombinant Constructs-pTZ57RT-*ompA* and pBudCE4.1-*ompA* were Validated Using Polymerase Chain Reaction

As illustrated in Fig. 2, the presence of 1112 bp band shows that the *ompA* has been successfully cloned into the pTZ57RT vector. This also validated using double digestion with *XbaI* and *BamHI* restriction enzymes. The presence of 2886 and 1112 bp bands was associated with pTZ57RT and *ompA* amplicon respectively (Fig. 3). Similarly, the result of colony PCR and *XbaI/BamHI* double digestion demonstrated that *ompA* has been successfully subcloned in pBudCE4.1 expression vector (Fig. 4).

Fig. 2 Amplification of 1112 bp from *ompA* gene using specific primers. Lane M, Lane 1 and Lane 2 represent the 100 bp DNA size marker, *ompA* amplicon and PCR negative control respectively

Fig. 3 Double digestion of recombinant pTZ57RT-*ompA* using *Xba*I and *Bam*HI restrictive endonucleases to validate the *ompA* cloning into the pTZ57RT vector. Lane M, Lane 1 and Lane 2 represent the 100 bp DNA size marker, pTZ57RT vector (2886 bp) + *ompA* fragment (1112 bp) and undigested pTZ57RT-*ompA* respectively

RT-PCR Demonstrated that HDF cells Received the *pBudCE4.1-ompA*

To confirm whether the *pBudCE4.1-ompA* has been successfully expressed in HDF cells, RT-PCR was performed on RNA extracted from transfected HDF cells using *ompA* specific primers. As indicated in Fig. 5, the presence of

1112 bp band showed that the cells uptake and express the construct.

The Recombinant Construct *pBudCE4.1-ompA* Efficiently Expressed OmpA Protein in HDF Cells

To reveal if the *pBudCE4.1-ompA* construct can express the OmpA protein, the HDF cells were transfected with recombinant vector for 48 h. The cell extract was then analyzed by SDS-PAGE to find the OmpA protein. As illustrated in Fig. 6, a 38 kDa band was observed on the PAGE which is similar to the predicted molecular weight of OmpA.

Discussion

This is the first experimental report to design a novel eukaryotic vector to clone the *ompA* gene from *A. baumannii* with a potential to be used as DNA vaccine. The OmpA was selected as immunogen, as the OmpA protein allocated the high degree of homology (~ 99%) among the all *Acinetobacter* species [15]. Additionally, it has demonstrated that OmpA is associated with multidrug resistant phenotype in *Acinetobacter* species [7, 8]. Our study can be considered novel as all previously reported immunization strategies against *Acinetobacter* species was achieved using either inactivated bacteria or purified synthetic outer membrane protein and other competent

Fig. 4 a The Cloning of *ompA* in pBudCE4.1 was validated by colony PCR using *ompA* specific primers. Lane M, Lane 1 and Lane 2 are 1 Kb DNA size marker, amplified *ompA* gene (1112 bp) from pBudCE4.1-*ompA* and negative control respectively. **b** Double digestion of recombinant pBudCE4.1-*ompA* using *Xba*I and *Bam*HI

restrictive endonucleases to validate the *ompA* cloning into the pBudCE4.1 vector. Lane M, Lane 1 and Lane 2 represent the DNA size marker, pBudCE4.1 vector (4595 bp) + *ompA* fragment (1112 bp) and undigested pBudCE4.1-*ompA* respectively

Fig. 5 a Integrity of RNA extracted from HDF cells. Total RNA (1 µg) extracted from HDF cells, was evaluated on 2% agarose gel. Lane M and Lane 1 present the 1 Kb DNA size marker and an extracted RNA sample respectively. The presence of bands corresponded to 28s rRNA, 18s rRNA and 5.8s rRNA confirmed acceptable quality of RNA extraction, **b** RT-PCR analysis of HDF

cells subjected to recombinant pBudCE4.1-*ompA* construct, using agarose gel electrophoresis and ethidium bromide staining. Lane M, 1 Kb DNA size marker; Lane 2: cells transfected with recombinant pBudCE4.1-*ompA* construct showing positive for *ompA* expression (1112 bp RT-PCR product band)

Fig. 6 SDS-PAGE analysis of *A. baumannii* OmpA protein. SDS-PAGE was used to analyze the expression of rOmpA. Recombinant vectors pBudCE4.1-*ompA* and pBudCE4.1 were transfected into HDF. All the samples were analyzed by SDS-PAGE, and the protein was stained with coomassie blue in the gel. Lane M: molecular mass marker in 10 kDa; Lane 1: whole cells lysate of the HDF with pBudCE4.1; Lane 2: the HDF including pBudCE4.1-*ompA*, 6 h after transfection; Lane 3–4, the whole cells lysate of the HDF contained recombinant pBudCE4.1-*ompA*, after 48 h; Lane 5: blank control (color figure online)

[1, 16–19]. On the other hand applying the eukaryotic vector to clone the *ompA* gene makes it promising construct to inject as the DNA vaccine in human. Such

eukaryotic expression vectors produce permanent and long lasting immunity in the body [20, 21]. Besides, our selected vectors has two multiple cloning sites makes it suitable to be used as a multivalent (divalent) DNA vaccine.

Generally, DNA vaccines are in interest due to their efficient and long lasting immunity [20]. They are easily manipulated and in spite of protein vaccines do not require specialized storage condition. Mass production of DNA vaccine can be easily achieved cost-effectively. It is also important to stress that DNA vaccines are considered safe and well tolerated as this generation of vaccine is not included with any weakened or dead pathogenic agents [20–22]. In fact, DNA vaccines are engineered plasmids carrying a desired gene to stimulate the specific immune response against a broad range of pathogens including bacteria, fungi or viruses [21]. The DNA vaccine has been also become attractive for scientists as many organisms are either non-culturable or cannot be attenuated as a vaccine. Besides, their ability to induce a variety of immune responses (both humeral and cellular), makes them as a powerful immunogenic tools [20–22].

Among different genus of *Acinetobacter* species, only *A. baumannii*, *A. nosocomialis* and *A. pittii* are the most clinically important types [23]. In comparison to all genus of *Acinetobacter*, *A. baumannii* is connected with the highest degree of mortality, morbidity and antimicrobial

resistance [24]. There is probably the reason that explains why *A. baumannii* is the favorite *Acinetobacter* genus for researchers to develop DNA vaccine than the others genus. Until now, limited studies were performed on *A. baumannii* to develop efficient immunization protocols. Inactivated whole cells, outer membrane proteins (OMPs) [25, 26], outer membrane vesicles (OMVs) [26], surface autotransporter Ata [18], biofilm-associated proteins [27], K1 capsular polysaccharide [28] and surface polysaccharide poly-*N*-acetyl- β -(1-6)-glucosamine (PNAG) [29] of *A. baumannii* are some effectual approaches to develop the vaccination protocol until now.

In study by Moriel et al., 62 antigens were identified in all *A. baumannii* species that could be used as potential vaccine targets and *ompA* was presented as one of these candidates [30]. In 2012, Luo and coworkers showed that rOmpA protein can produce active and passive immunization following the injection into the diabetic mice suffering from *A. baumannii* infection although serum resistant was not achieved [31]. In another study by Lin et al., they showed that OmpA protein can protect the *A. baumannii*-induced infection in mice through the induction of IL-4 and INF- γ signaling pathways [32]. Similarly, Alzubaidi et al. demonstrated that coinjection of OmpA protein with chitosan nanoparticle as adjuvant increased the humoral immunity and appraised the efficiency of the vaccination against *A. baumannii* in mice [33].

Although no DNA vaccine has been constructed for *A. baumannii* until now, our study can be considered promising. However, it should not be ignored the oncogenic potential of such recombinant molecules following a random integration into the host genome. Additionally, the immunogenic properties will be increased if the vaccine is used with adjuvants like IL-4 or GMSF. These are the issues that should be addressed in future experiments.

Conclusion

In this study the *ompA* gene of *A. baumannii* was successfully cloned into the pBudCe4.1 eukaryotic expression vector. The capacity of vector to express OmpA protein was confirmed in HDF cells using RT-PCR. So we suggest pBudCE4.1-*ompA* construct as a DNA vaccine candidate against *A. baumannii* although further experiments are needed.

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Authors Contribution HA; literature review, manuscript writing and data collection. AD; literature review and manuscript revision, MK; manuscript revision, MB; manuscript revision and data collection and MJ; literature review.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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