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Escherichia coli'de *Acinetobacter baumannii* OccAB1 Porin Proteininin Klonlanması, Ekspresyonu ve Saflaştırılması

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ÖZ

Acinetobacter baumannii; toprakta, suda, insan ve hayvan vücutlarının yüzeyleri gibi farklı habitatlarda serbest yaşayan gram negatif bir organizmadır. Ancak zamanla patojenik bir yaşam tarzına adapte olarak tüm dünyada yoğun bakım ünitelerinde enfeksiyonlara ve ölümlere neden olmaktadır. Antibiyotik direncinde porin proteinleri bu bakteride temel rol oynamaktadır. Porinler ve dışa akış pompalarının aşırı ekspresyonu; ilaç direncini artırmada ve tedavinin etkisiz hale getirilmesinde katkıda bulunan faktörler olarak tespit edilmiştir. Bu sebeple antibiyotik geçişlerinin *in vitro* olarak çalışılabilmesi için öncelikli olarak bu proteinlerin saflaştırılması gerekmektedir. Saflaştırma işleminin ardından elde edilen bu porin proteini ile çift katmanlı lipit tabakalarda antibiyotiklerin geçişleri araştırılacaktır. Sonuç olarak bu çalışmada, *A. baumannii* OccAB1 porin proteini klonlandı ve *E. coli*'de başarılı bir şekilde eksprese edildi.

Cloning, Expression, and Purification of *Acinetobacter baumannii* OccAB1 Porin Protein in *Escherichia coli*

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ABSTRACT

Acinetobacter baumannii is a free-living gram-negative organism in different habitats such as soil, water, and surfaces of human and animal bodies. However, adapting to a pathogenic lifestyle over time causes infections and deaths in intensive care units worldwide. Porin proteins play a fundamental role in antibiotic resistance in this bacterium. Overexpression of porins and efflux pumps have been identified as contributing factors in increasing drug resistance and inactivation of therapy. For this reason, to study antibiotic transitions *in vitro*, these proteins must be purified first. With this porin protein obtained after purification, the passage of antibiotics in bilayer lipid layers will be investigated. In conclusion, in this study, *A. baumannii* OccAB1 porin protein was cloned and successfully expressed in *E. coli.*

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1. Introduction

Proteins are extremely complex molecules in terms of their structure and functions. For this reason, unlike many pharmaceuticals, they cannot be synthesized chemically. Therefore, proteins are synthesized in biological processes, often inside host cells of different types from their origin. These synthesized proteins are called 'recombinant proteins' because of the recombination or rearrangement of the DNA encoding them (Overton, 2014).

Expression and purification of recombinant proteins allow for detailed study of proteins. In this way, the expression of many uncharacterized proteins in large amounts has been achieved, and it has been possible to study biochemical, structural, and functional aspects (Assenberg et al., 2013; Joshi and Jain, 2017).

Prokaryotic organisms, yeasts, insects, and mammalian cell systems are mostly used to produce recombinant proteins. The most widely used prokaryotic cell for recombinant protein production in *Escherichia coli* as a long and safe history of use in laboratories and industry (Huang et al., 2012; Joshi and Jain, 2017).

Because it is among the first organisms that are physiologically and metabolically well-characterized and whose whole genome sequence is known (Blattner et al., 1997), it is also a preferred organism as it provides several advantages such as rapid growth, low cost, ease of large-scale culture, and genetic manipulation (Demain and Vaishnav, 2009).

The vectors used and the host in recombinant protein production is extremely important. Because the promoter in which the expression of the relevant recombinant gene is regulated is important for the expression system. Commonly used promoter systems are based on T7 RNA polymerase (Studier, 1991). This system is under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter. In the absence of IPTG, LacI binds to this promoter system, and T7 RNA polymerase is not synthesized. Upon addition of IPTG, LacI is detached from the lac promoter region, and the T7 RNA polymerase gene is amplified and converted to the product (Peränen et al., 1996). Many proteins can be produced recombinantly in this way.

Porin proteins are among the proteins that have a very important role in protecting gram-negative bacteria against environmental stresses. These proteins in the outer membrane are in direct contact with the external environment due to their location (Nikaido, 2003). They are among the reactions of the cell for protection against changes in the environment. They have very important roles in protecting the cell against external chemical and physical factors such as oxidative stress, pH, osmolarity, toxic chemicals (Darcan et al., 2003; Pagès et al., 2008; Darcan et al., 2009; Darcan, 2012; Darcan and Aydin, 2012; Choi and Lee, 2019). In addition, it is known that bacteria play a very important role in resistance to chemicals such as antibiotics (Nikaido and Vaara, 1985; Lee et al., 2013).

Acinetobacter baumannii is the most active species among the bacteria that cause hospital infections called ESKAPE. This bacterium is the biggest factor in deaths caused by disease agents transmitted in

intensive care units (Pagès et al., 2008; Rice, 2008, 2010; Santajit and Indrawattana, 2016). Porin proteins have a very important role in the antibiotic resistance of these bacteria (Vila et al., 2007).

It has been determined that there are many porins, especially the OccAB1 porin, in *A. baumannii* (Borneleit and Kleber, 1991; Bhamidimarri et al., 2019; Rasooli et al., 2020). These porins have important roles in antibiotic resistance. The channel diameters and charges of the porin proteins have a control role in the selective passage of substances from the outside (Vila et al., 2007; Tokuda, 2014; Bhamidimarri et al., 2019; Uppalapati et al., 2020).

The number of porins and their small diameters in *A. baumannii* reduce the permeability of the outer membrane compared to that of other gram-negative. Therefore, the outer membrane of *A. baumannii* is less permeable than *Escherichia coli* (Obara and Nakae, 1991).

Therefore, it is necessary to investigate the roles of *A. baumannii* porins in antibiotic resistance and to reveal their channel properties. In particular, it should be revealed how the mutations that will occur will reveal changes in antibiotic resistance. Therefore, it is important to obtain it recombinantly to work with this porin.

In this study, OccAB1 porin expression in *E. coli* was performed by cloning from *A. baumannii* (ATCC 19606) to determine the roles of OccAB1 porin in antibiotic resistance. Antibiotic transitions from the lipid bilayer with recombinantly obtained this porin protein will be examined. Therefore, this study carried out the cloning and expression of the *A. baumannii* OccAB1 porin in *E. coli*.

2. Materials and Methods

2.1. Cloning of the *occAB1(oprD)* gene in *Acinetobacter baumannii*

A. baumannii reference strain ATCC 19606 was incubated in 5 ml LB broth medium at 37 ^{o}C for 18 hours with shaking, and after incubation, the culture was centrifuged at 12000 g for 5 minutes at 4 ^{o}C. The pellet obtained after centrifugation was washed twice with PBS and homogenized with 600 µl extraction buffer (100 mM EDTA, 100 mM Tris-HCl pH 8.0). After adding 6 µl of lysozyme (3 mg/ml) to the suspended cells, it was incubated at 37 ^{o}C for 15 minutes, then 60 µl of 10% sodium dodecyl sulfate (SDS) and 9 µl of proteinase K (150 µg/ml) were added onto the cell suspension. It was incubated for another 30 minutes at 37 °C. Then, 90 µl of 10% CTAB and 60 µl of 10 M NaCl stock were added and mixed. After that, it was incubated for 10 minutes in a 65 ^{o}C water bath, kept in liquid nitrogen for 1-2 minutes, frozen, and put back at 65 ^{o}C. After repeating this process 3 times, 900 µl of phenol:chloroform: ısoamyl alcohol (25:24:1) was added and centrifuged at 13000 g at 4 ^{o}C for 5 minutes. After the upper liquid phase was removed, 1 volume of phenol:chloroform: ısoamyl alcohol was added and this process was continued until the protein intermediate phase was reduced. Then, 80 µl of sodium acetate (3M, pH 4,8), 8 µl of $MgCl_2$ (1M), and 480 µl of isopropanol were added and centrifuged at 13000 g at 4 ^{o}C for 30 minutes. 70% alcohol was added to the pellet and centrifuged again for 15 minutes at 13000 g at 4 ^{o}C. Afterward, the alcohol was removed, and the pellet was dried

at room temperature, resuspended in 50 µl of diethylpyrocarbonate (DEPC) water, and the DNA was stored at -20 °C.

These obtained DNA samples were used for amplification of the *occAB1(oprD)* gene. For this purpose, a primer was designed with the PRIMER3 program for the OccAB1(OprD) porin protein of *A. baumannii,* suitable for ligation with the pLATE51 vector (Thermo Scientific K1251–N-terminal His-tag/EK). For the designed primer to form sticky ends, specific sequences to the vector were added.

Table 1. *occAB1(oprD)* primers of *Acinetobacter baumannii*

pLATE51 *occAB1(oprD)* Forward primer	5'GGTGATGATGATGACAAGATGCTAAAAGCACAAAAACTTA3'
pLATE51 *occAB1(oprD*) Reverse primer	5'GGAGATGGGAAGTCATTAGAATAATTTCACAGGAATATCT3'
Plasmid Forward primer	5'TAATACGACTCACTATAGGG3'
Plasmid Reverse primer	5'GAGCGGATAACAATTTCACACAGG3'

2.2. PCR reaction

Using primers designed for the *A. baumannii occAB1*(oprD) gene indicated in Table 1, the *occAB1*(oprD) gene was amplified from total DNA by PCR reaction. For each PCR reaction, a mixture was made containing 70 µl of sterile water, 10 µl of 10X ThermoPol Reaction Buffer, 2.5 µl of 10 mM dNTP Mix, 6 µl of 2 mM $MgCl_2$, 0,5 µl of each 10 µM primer stock, and overlaid 0,5 µl (0,25 U) of Taq polymerase was added. Then template DNA (1 µl) was added to the 0.2 ml PCR tube, and 99 µl of the prepared PCR mixture was added. After mixing, the sample was minispinned for 5 s and centrifuged for 5 s. PCR was performed as follows: pre-denaturation at 94°C for 3 min followed by 30 cycles; Denaturation at 94°C for 1 minute, annealing at 67°C for 1.5 minutes, and elongation at 72°C for 1.5 minutes was performed. The amplified product was electrophoresed on 1% agarose and then purified.

2.3. Purification of PCR product

50 µl of TE-saturated phenol and 50 µl of chloroform: isoamyl alcohol (24:1) were added to the 100 µl PCR product obtained as a result of PCR, and the samples were vortexed and centrifuged at 12000 g for 5 minutes in room temperature. After centrifugation, the supernatant was taken, and an equal volume of chloroform: isoamyl alcohol (24:1) was added and then centrifuged at 12000 g for 2 minutes at room temperature. After centrifugation, the supernatant part was taken, and 5 M NaCl at 1/25 of its volume and 100% EtOH at 2 times its volume were added. Then, it was incubated at -80°C for more than 30 minutes, and after incubation, it was centrifuged at 12000 g for 10 minutes at 0°C. After centrifugation, the upper liquid phase was discarded, and the precipitate was taken. The

precipitate was treated with 70% alcohol and then centrifuged at 12000 g for 5 min at 0 °C. Then, it was left to dry for about 5 minutes at room temperature, and 10 µl of pH 7.5 TE (for 100 µl PCR product) was added to the dried precipitate and stored at -20°C.

2.4. Ligation

After purification, ligation of the *occAB1(oprD) gene* PCR product of *A. baumannii* into the pLATE51 vector was performed. The ligation step to the Alicator pLATE51 vector was performed as follows; Add 1 µL of PCR product, 2 µl of 5X LIC buffer, 1 µl of T4 DNA polymerase (1 µ/µl), and 6 µl of pure water and incubate at room temperature for 5 minutes. Then, after adding 0.6 µl of 0.5 M EDTA and 1 µl of pLATE51 vector to the reaction mixture, ligation was performed by incubating for 5 minutes at room temperature.

2.5. Transformation

To prepare a pre-culture for transformation, the *E. coli* W3110 strain was incubated in an LB broth medium at 37 °C overnight. After the incubation, 2 M Mg^{+2} 100 µl and 50 µl of the prepared pre-culture were added onto a sterile 9.9 ml SOB medium and incubated at 37 ^{o}C with shaking until the OD_{600} value was 0,3. After incubation, the cells were transferred to the centrifuge tube and cooled on ice for 5 minutes, and then centrifuged at 12000 g for 5 minutes at 4 °C. Afterward, the supernatants were removed, and 1 ml of 0.1 M cold $CaCl_2$ was added to the pellet, the cells were suspended and incubated on ice for 10 minutes. It was centrifuged again at 12000 g for 5 min at 4 ^{o}C. The supernatant was removed again, and 0.1 M cold 200 µl $CaCl_2$ was added per tube, and then the pellet was resuspended and incubated on ice for 30 min. Then, 10-100 ng of a plasmid containing the *occAB1(oprD)* gene at two different concentrations was added to 200 µl of competent cells in the centrifuge tube and incubated on ice for 45 minutes. Then, the cell suspensions were transferred to glass tubes, and firstly heat shock at 42 ^{o}C for 2 minutes and then cold shock on ice for 2 minutes was applied. Then, 0.8 ml of SOC medium was added to the shocked cells and incubated at 37 °C for 60 minutes with shaking. After incubation, the recombinant cells were centrifuged at 5000 g for 2 minutes at 4 °C and the supernatant was discarded, and the cells were suspended by adding 100 µl of SOC medium to the pellet. The suspended cells were spread on LB agar containing ampicillin antibiotic and incubated overnight at 37 °C. After incubation, colony PCR was performed with the cells grown in the petri dish.

2.6. Colony PCR

The colony PCR method confirmed the recombinant cells obtained after the transformation process. Colony PCR was performed using the plasmid primers indicated in Table 1 for validation. For each PCR reaction, a mixture was made containing 10X ThermoPol Reaction Buffer 1 µl, 0,2 µl of 10 mM dNTP Mix, 0,6 µl of 2 mM $MgCl_2$, 0,5 µl of each 10 µM primer stock of each primer, 0,05 µl (0,25 U)

of Taq polymerase, and 6,15 µl of sterile water. 1 µl of the suspended cell obtained from a single bacterial colony was added to 0.2 ml PCR tubes as a template. Followed by the addition of 9 µl of PCR master mix. PCR was performed as follows: pre-denaturation at 94°C for 3 min followed by 30 cycles; Denaturation at 94°C for 1 minute, annealing at 67°C for 1.5 minutes, and elongation at 72°C for 1.5 minutes was performed. The amplified product was electrophoresed on 1% agarose.

2.7. Agarose gel electrophoresis

PCR products were checked with 1% agarose. For this, 1 g of agarose and 100 ml of 1x TBE were used, and this mixture was heated in the microwave and completely dissolved. Then, 1 µl of EtBr from 10 mg/ml EtBr stock was added, and the prepared agarose gel was poured into the tank of the electrophoresis device. After the gel was frozen, 5 µl of the sample was mixed with 1 µl of loading dye and loaded into the gel.

2.8. Purification of *Acinetobacter baumannii* OccAB1(OprD) protein from recombinant *Escherichia coli* by Ni-NTA method

Recombinant *E. coli* cells containing the *A. baumannii occAB1 (oprD)* gene were inoculated into 5 ml of LB broth medium and then pre-cultured by incubating overnight in a 37 ºC 160 rpm shaking incubator. The next day, 2 ml of the pre-culture was added to the flask containing 100 ml of LB broth, and incubation was continued at 37 ºC with shaking at 160 rpm until the OD_{600} value reached 0.6-0.8 absorbance. When the OD_{600} value reached 0.6-0.8 absorbance, IPTG was added to the culture at a final concentration of 0.5 mM, and incubation with shaking was continued for 6 hours to induce the plasmid. After incubation, 100 ml of bacterial cells were centrifuged at 12000 g for 5 minutes at 4 °C. The supernatant was discarded, and the bacterial pellet was washed with 1x PBS and centrifuged again at 12000 g for 5 minutes at 4 °C. After centrifugation, 12 ml of lysis buffer (50mM $NaH_2PO_42H_2O$, 300 mM NaCl, 10 mM imidazole, 1% Tween 20, and 0.5% beta-mercaptoethanol) was added to the pellet, and sonication was performed on ice 5-6 times at 6 amplitudes. After sonication, the cell suspension was centrifuged at 8000 g for 5 min at 4 °C. Then, the supernatant was taken into a clean test tube, 1 ml of Ni-NTA solution was added, and incubated at 9 rpm for 4 hours at 4 ^oC with shaking. After incubation, the supernatant was centrifuged at 4000 g for 1 minute at 4 ^oC. After centrifugation, the supernatant was taken into a clean test tube and stored at -20 °C. Then, 850 µl of wash buffer (50mM $NaH_2PO_42H_2O$, 500 mM NaCl, 20 mM imidazole, 10% Glycerol, and 0.2% Triton X 100 (or 0.5% Tween 20)) was added to the pellet. It was then centrifuged at 4000 g for 1 min at 4 ^oC. This process was repeated 3 times. After the last washing process, elute buffer (50mM $NaH_2PO_42H_2O$, 500 mM NaCl, 400 mM imidazole, 10% Glycerol, and 0.2% Triton X 100 (or 0.5% Tween 20)) was added to the pellet and the pellet was centrifuged again at 4000 g for 1 minute at 4 ^oC. This process was also repeated 3 times. All samples obtained were stored at -80 °C for western blot.

2.9. Western blot

In Western blot analysis, two numbers of 12,5% SDS gel were used to the separating the proteins. After all the samples were denatured at 95 ºC for 8 minutes by adding Laemmli buffer, they were loaded equally on both gels and by adding the running buffer on the SDS gel, the samples were first run at 70V for 10 minutes and then at 100-120V until the gel was finished. Then, one of the gels was stained with coomassie brilliant blue for 10 minutes and washed with a destaining buffer as a control. This process was done for pre-western blot control purposes. If the proteins were isolated and equally loaded, the transfer of proteins to the nitrocellulose membrane was carried out with the other gel. After the transfer process, the nitrocellulose membrane was stained with Ponceau S dye to check whether the transfer took place correctly. After the transfer process, the nitrocellulose membrane was blocked with 5% milk powder at room temperature for 1 hour. After blocking, the nitrocellulose membrane was incubated with diluted rabbit monoclonal His-tag antibody (1:1000) overnight at 4°C. Then, the membrane was washed with 1X TBST buffer for 3 sets for 10 minutes each and placed on the anti-rabbit secondary antibody. Here, after incubation for 1 hour at room temperature, the membrane was washed again with TBST buffer for 3 sets of 10 minutes each. Finally, the membrane was washed once with TBS and left in the dark for 2 minutes by adding the chemiluminescence reagent ECL to the membrane for imaging and the bands were visualized in GBOX-Chemi-XRQ, Syngene.

3. Results

3.1. Replication of the *occAB1(oprD)* gene in *Acinetobacter baumannii*

The *occAB1(oprD)* gene was PCR amplified from genomic DNA isolated from *A. baumannii* ATCC 19606 (Figure 1). After amplification, these gene regions were purified as shown in Figure 2. After purification, the *A. baumannii occAB1(oprD)* gene region was ligated with the pLATE51 plasmid. Subsequently, the obtained recombinant cells were confirmed by colony PCR using the primers in Table 1. PCR results of the *occAB1(OprD)* gene region with a size of 1317 bp are given in Figure 3 and validation results with plasmid primers are given in Figure 4. After the transformation process, SDS gel electrophoresis was performed to observe the addition of the 6x histidine tail used in the purification of the OccAB1(OprD) porin protein. The SDS gel was stained with coomassie brilliant blue to see if protein isolation and Ni-NTA purification were successful. In the lysate, in which the cell is successfully lysed, all the proteins to which the nickel beads are attached and to which they are not bound are also present in the flow-through. Other proteins were tried to be removed in washes and elutes, and OccAB1(OprD) protein remained pure. In the 3rd and 4th wells, which are the last step of the elutes, there is the purified form of the OccAB1(OprD) protein (Figure 5). In addition, the OccAB1(OprD) porin protein was confirmed by western blot (Figure 6).

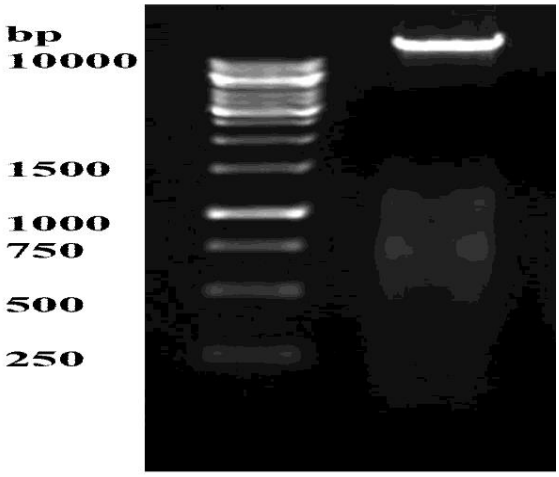

Figure 1. Genomic DNA isolation from *Acinetobacter baumannii* (ATCC19606)

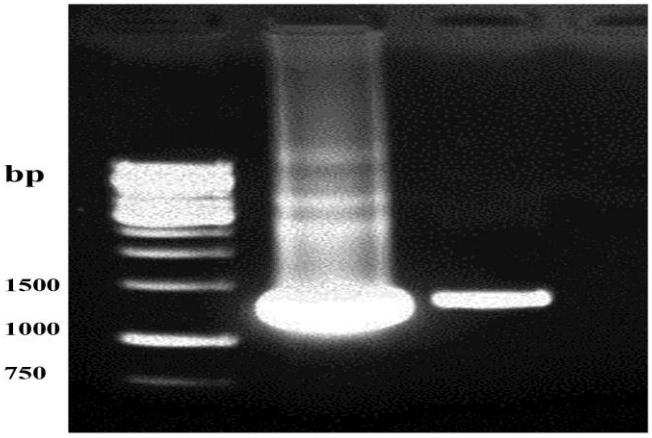

Figure 2. Purification result of *Acinetobacter baumannii occAB1(oprD)* gene PCR product

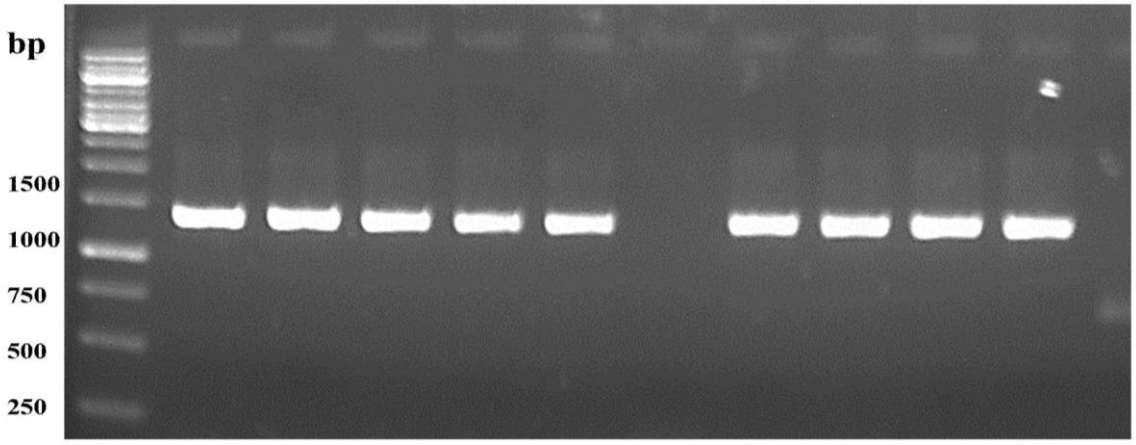

Figure 3. PCR result of *Acinetobacter baumannii occAB1(oprD)* gene in recombinant cell *Escherichia coli. (All wells are PCR results using occAB1(oprD)* gene *primers of recombinant cells in petri plates)*

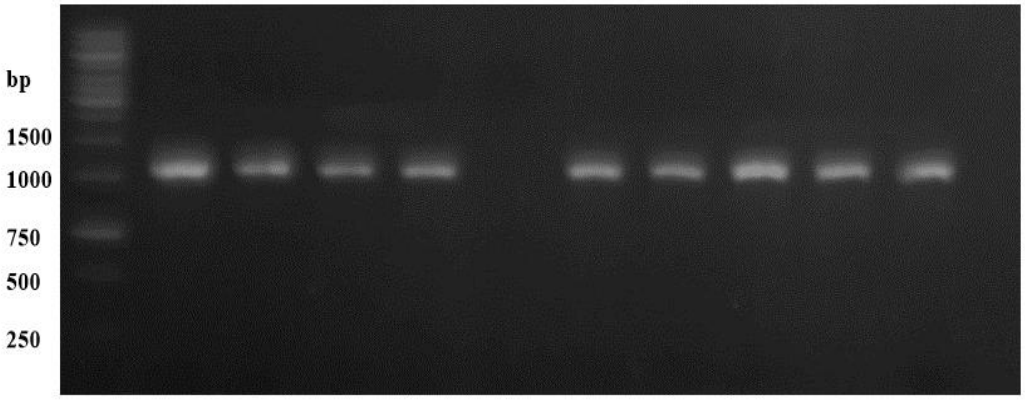

Figure 4. Confirmation of the *occAB1(oprD) gene* in recombinant *Escherichia coli* strains with plasmid primer *(All wells are PCR results using plasmid primers of recombinant cells in petri plates)*

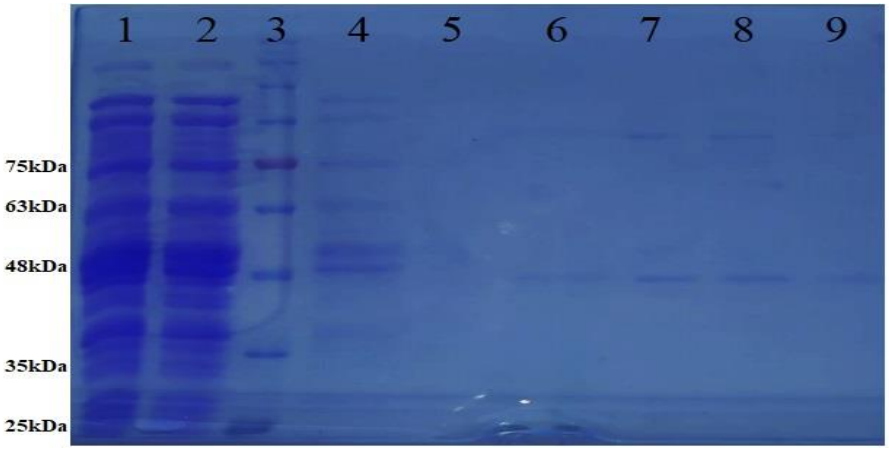

Figure 5. Ni-NTA purification result of *Acinetobacter baumannii* OccAB1(OprD) protein in recombinant cell *Escherichia coli*
(1. Lysate, 2. Flow-through, 3. Marker, 4. WashI, 5. WashII, 6. Elute I, 7. Elute II, 8. Elute III, 9. Elute IV).

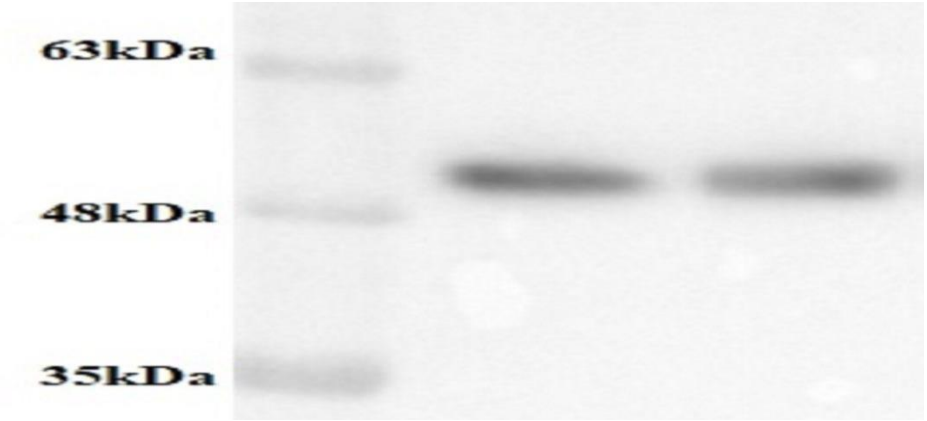

Figure 6. Representation of OccAB1 (OprD) protein of *Acinetobacter baumannii* in recombinant *Escherichia coli* with anti-his tag antibodies in western blot

4. Discussion

Acinetobacter baumannii is a gram-negative organism that causes hospital infections and serious health problems due to multi-drug resistance (Esterly et al., 2011; Abbott et al., 2013). Resistance development in *A. baumannii* is mostly based on outer membrane proteins. Outer membrane proteins participate in various functions that help bacteria withstand harsh environmental conditions and combat the threat posed by antimicrobial compounds. The absence of some porins with wide channels, such as OmpC and OmpF in *A. baumannii*, causes low permeability in the outer membrane, making the bacteria resistant to many antibiotics (Sugawara and Nikaido, 2012). *A. baumannii* has many outer membrane proteins including OmpA, CarO, AbuO, Omp 33-36 kDa, TolB, DcaP, CadF, Oma87/BamA, OprD-like OMPs (OccAB), OprF NmRmpM (Huang et al., 2012; Park et al., 2012; Srinivasan et al., 2015; Lee et al., 2017; Bhamidimarri et al., 2019). Of these, OccAB porins are channels of great importance for the entry of small molecules collectively in *A. baumannii*, and *A. baumannii* has a total of five Occ (OM carboxylate channel) proteins. These are OccAB1 (OprD), OccAB2 (HcaE), OccAB3 (VanP), OccAB4 (BenP) and OccAB5 (Segura et al., 1999; Clark et al., 2002; Smith et al., 2003; Dupont et al., 2005). OccAB1 is the only Occ protein that has so far been biochemically characterized from *A. baumannii*. One study reported the involvement of OccAB1 in the intracellular uptake of imipenem (Dupont et al., 2005; Zahn et al., 2016). Many clinical studies have identified the differential expression of outer membrane proteins in antibiotic-resistant *A. baumannii* strains, revealing their role in providing resistance(Vashist et al., 2010; Vashistt et al., 2011; Mostachio et al., 2012). In this study, OccAB1(OprD) porin in wild type *A. baumannii* ATCC 19606, the permeability to antibiotics was studied. Firstly, this porin was expressed and purified in *E. coli.* pLATE51 vector and Ni-NTA purification method were used to express and purify OccAB1 porin successfully. Many studies in the literature mention that the Ni-NTA purification method is an extremely successful method (Verma et al., 2009; He et al., 2021; Nurjayadi et al., 2021).

In this study, OccAB1 porin protein from *A. baumannii* ATCC 19606 strains was cloned and expressed in *E. coli.* In the continuation of the study, experiments were carried out on the passage of antibiotics through the lipid layer, and new information was obtained on the antibiotic permeability and drug resistance of porins.

5. Conclusion

Porins are control sites for the passage of antibiotics into the periplasmic space. Loss of porin transport channels alters the efficacy or uptake of antibiotics and causes significant hospital-acquired infections. For this reason, isolation and purification of porins is extremely important for detailed and individual analysis of each porin. Thus, with the resolution of the porin-antibiotic relationship, contributions are made at the molecular level to drug resistance studies.

In this study, wild-type *A. bauminnii* ATCC 19606 the *occAB1* gene was successfully amplified using the PCR method. This gene region obtained was cloned into the pLATE51 vector and successfully

transformed into *E. coli* W3110 bacteria. Then, the constitutive expression of OccAB1 protein from this recombinant strain was purified and purified using the Ni-NTA method. The His-tag antibody confirmed the accuracy of the obtained protein. In the continuation of the study, the passage of antibiotics through the bilayer lipid layer will be analyzed with this purified protein *in vitro*.

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Conflict of Interest

There is no conflict of interest between the authors.

Author's Contribution

The authors declare that they have contributed equally to the article.

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