Molecular uptake of antibiotics through the silent chitoporin from *Escherichia coli* (*Ec*ChiP)

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Abstract: *Ec*ChiP is a monomeric protein channel found in the outer membrane (OM) of *Escherichia coli* (*E. coli*) and used for the transport of small chitooligosaccharides across the OM. In this study, we identified antibiotic transport through the *Ec*ChiP-reconstituted in lipid membrane. The antibiotics that can inhibit bacterial growth were further chosen for evaluating their specific interactions with *Ec*ChiP channel. The results show that 2 mM of gentamycin, minocycline, and tigecycline could occlude the ion flow through the *Ec*ChiP channel, indicating that these antibiotics could enter and interact with the channel lumen. Crystallizations of *Ec*ChiP in complex with gentamycin, minocycline, and tigecycline were grown in the optimized condition G8 (33% PEG400, 0.1 M sodium chloride, 0.1 M MES, pH 6.5) from MemGold1™ and the condition E10 (33% PEG400, 0.23 M sodium chloride, 0.05 M sodium acetate, pH 4.5) from MemGold2™ screening kits. The crystals have a long tetragonal shape with estimated sizes of 300 – 500 μm. The predicted structure of *Ec*ChiP in complex with minocycline, gentamycin, and tigecycline indicated that all three antibiotics occupied the constriction zone of the protein pore. Understanding the antibiotics-*Ec*ChiP interactions may suggest an effective approach to design for novel anti-microbial agents against infectious strains of *Escherichia coli*.

Keywords: antibiotic resistance, chitoporin, *E. coli*, single channel

1. Introduction

Escherichia coli (*E. coli*) is a Gram-negative bacterium (1) commonly found in soil, manure, and water, which are rich in carbon sources, and *E. coli* could easily grow on glucose-enriched nutrients (2). Several strains of *E. coli* are pathogens that can cause cystitis and extraintestinal illnesses, including pneumonia, bacteremia, and abdominal infections, such as spontaneous bacterial peritonitis (3). Normally, *E. coli* infections can be treated by many types of antibiotics but nowadays the highly resistant rate of *E. coli* has been reported (4, 5).

E. coli's cell wall contains double lipid membranes with a thin layer of periplasmic space between them (6). There are protein components that are commonly found in the outer membrane, which are used for the transport of small nutrients, namely porins. Normally, small nutrients with limited MW of < 600 Da could enter the bacterial cells by general diffusion porins (7–9). However, there are certain hydrophilic molecules, which cannot pass through general diffusion porins, such as chitooligosaccharides or other oligosaccharides. These molecules require solute-specific porins. For example, chitooligosaccharides can be transported through the cell wall of *E. coli* through chitoporin, recently

identified according to its specificity to the sugar substrate as *Ec*ChiP. *Ec*ChiP belongs to one of the OprD-like porin family, which is a monomeric OM channel and responsible for chitooligosaccharide uptake with chitopentaose/chitohexaose being the most preferred substrates (2, 10). However, other physiological functions of *Ec*ChiP have not been published. In recent years, outer membrane porins are not only responsible for nutrient uptake, but they also play an important role in the permeability of antibiotics across OM, and the ability of antibiotics to permeate through the OM also define a level of drug resistance of the bacteria. For example, kanamycin was reported to be able to pass through OmpF and OmpC channels (11). In this study, we would like to demonstrate that chitoporin can be used as a molecular gateway for sugar-based antibiotics to enter the *E. coli* cells.

We first evaluated the antimicrobial susceptibility of *E. coli* by agar disc assay. The tested antibiotics that can inhibit bacterial growth were then used for screening of molecular interactions with the membrane embedded *Ec*ChiP channel using black lipid membrane (BLM) reconstitution technique. After that, the antibiotics that interacted with the protein channel were used for co-crystallization. In addition, the results obtained from BLM experiments were used for further prediction by GOLD docking to identify the molecular basis of the protein-ligand complexes (12).

2. Materials and Methods

2.1 Antimicrobial susceptibility assays

Antimicrobial susceptibility of *E. coli* DH5α was investigated by Minimum Inhibitory Concentrations (MIC) and agar disc diffusion assay (13). MIC values were determined by the Mueller-Hinton (MH) two-fold dilution method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (14). *E. coli* DH5α cells were grown in MH media agar for 16 hours and then single colonies were picked and grown in MH media at 37 °C overnight, 16 hours. Then, cells were measured and diluted to OD_{600} = 0.1 by 0.9% NaCl. For each antibiotic, a 96 wells-microplate was filled by 200 μL of medium contained antibiotics with 18-serial dilutions of antibiotic concentration set from 2,048 $\mu g \cdot mL^{-1}$ to 0.015625 $\mu g \cdot mL^{-1}$. Then, 10 μL of the diluted cell suspension was added into the microplate and incubated overnight at 37°C. MIC values were evaluated in comparison with the breakpoints for *E. coli* as recommended by the European Committee on Antimicrobial Susceptibility Testing EUCAST (www.eucast.org/clinical_breakpoints/). For agar disc diffusion assay, all antimicrobial agents were used for the susceptibility tests. *E. coli* DH5α cells were grown in MH media agar for 16 hours, and then single colonies were picked and grown in MH media at 37 °C overnight for 16 hours. Then cells were measured and diluted to OD_{600} = 0.5 by 0.9% NaCl. Bacterial solutions were swabbed on MH media agar twice and discs were pressed on the plate immediately. After that, different antibiotics were dropped on to sterilize antibiotic discs (0.6 mm diameter, Whatman, UK) by varying the amount of the antibiotic to 10, 20, 30 and 40 μg. The clear zones were observed and measured after incubation for 16 hours at 37 °C.

2.2 Protein expression and purification

For expression and purification of the OM-expressed *Ec*ChiP, the recombinant plasmid, pET23d(+)/*EcChiP*, was transformed in the *E. coli* BL21(DE3) Omp8 Rosetta strain and the overnight culture of transformed cells was transferred to Luria-Bertani (LB) broth containing 100 $\mu g \cdot mL^{-1}$ ampicillin and 25 $\mu g \cdot mL^{-1}$ kanamycin and grown at 37 °C. During the exponential growth phase (OD_{600}~0.6-0.8), *Ec*ChiP expression was induced by 0.4 mM isopropyl thio-β-D-galactoside (IPTG). After 6 hours of further incubation at 37 °C, the cell pellet was harvested by centrifugation at 8000 rpm for 15 min at 4 °C. For extraction, the cell pellet was re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$), containing 0.1% (v/v) Triton X-100, 10 $\mu g \cdot mL^{-1}$ RNase A and 10 $\mu g \cdot mL^{-1}$ DNase

I. Cells were broken on ice with a high-speed ultrasonic processor (Cole-Parmer Instrument, Vernon hills, Illinois, USA) for 30 min, then 2% (w/v) SDS was added to the cell suspension, which was further incubated at 50 ˚C for 60 min with 300 rpm using thermomixer. The SDS solution was separated by ultracentrifugation at 22,000 rpm, 25 ˚C for 60 mins. Next, the cell pellets with *Ec*ChiP were washed by 0.125% (v/v) Octyl-POE in 20 mM phosphate buffer pH 7.4, and the solution was incubated at 37°C, 300 rpm for one hour. After that, the cell components were removed by ultracentrifugation at 44,000 rpm at 4 ˚C for 45 mins. The pellet containing *Ec*ChiP in the membrane fraction was extracted by 2% (v/v) lauryldimethylamine oxide (LDAO) in 20 mM phosphate buffer pH 7.4 which using homogenizer potter and incubated at 37 °C, 300 rpm for 16 hours before collecting by ultracentrifugation at 40,000 rpm. For purification, *Ec*ChiP solution was purified by **i**on-exchange chromatography using a Hitrap Q HP prepacked column which was connected to ÄKTA™ prime plus FPLC system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The proteins were eluted with a linear gradient of 0-100 M KCL in 20 mM phosphate buffer, pH 7.4, containing 0.2% (v/v) LDAO. The purity of the *Ec*ChiP peak were verified by SDS-PAGE. Finally, fractions containing *Ec*ChiP were purified by size exclusion chromatography using a 26/100 Sephacryl S-200^{HR} column connected to ÄKTA™ prime plus FPLC system. The *Ec*ChiP protein was eluted by 10 mM HEPES pH 7.4, 100 mM NaCl with 0.2% LDAO and verified by SDS-PAGE. After that, the highly purified *Bps*Omp38 fractions were pooled together and used for crystallization and Black lipid membrane (BLM) experiments.

2.3 Single channel electrophysiology

Permeation of antibiotics through *Ec*ChiP was measured by black lipid membrane (BLM) experiments. The antibiotic entrance through the single channel was carried out following previous studies (15, 16). Briefly, a Teflon film with a small aperture of 50-100 μm was separated the two-cell compartment (designated *cis* side and *trans* side) and pretreated the aperture by 2 μL of 1% (v/v) hexadecane in hexane with three times, the chambers were filled with 1 M KCl in 20 mM HEPES, pH 7.4 as the electrolyte solution. Ion current was measured by Ag/AgCl electrodes, one of the electrodes was used as ground (*cis*) and the other one was connected (*trans*) to the Axon 1550® amplifier (Molecular Device, Germany). Then, lipid bilayer was formed between two chambers by adding 5 μL of 5 mg·mL^{-1} DPhPC (1,2-Diphytanoyl-sn-glycero-3-phosphatidyl-choline) lipid in pentane into each side of the chamber followed by raising and lowering the solution. After the stable planar lipid bilayer formation, 100 μg·mL^{-1} protein was always added into the cis chamber gradually increased by adding 1 – 10 μL until a single channel insert into the lipid bilayer. Antibiotics translocation of *Ec*ChiP were investigated after the *Ec*ChiP channel was fully opened and added 2 mM of antimicrobial agents. All traces were recorded for 2 minutes at ±100 mV potentials and the results were analyzed by *Clampfit 11.1*. (all purchased from Molecular Devices, Sunnyvale, CA)

2.4 Antibiotics-EcChiP complex crystallization

For co-crystallization of *Ec*ChiP with antibiotics, the freshly purified *Ec*ChiP was exchanged buffer to 10 mM HEPES pH 7.4, containing 100 mM LiCl contained 0.4**%** (v/v) C_8E_4 (tetraethelene glycol monooctyl ether) using Amicon concentrator (30 kDa molecular weight cut off). Next, *Ec*ChiP with a concentration of 10 - 12 mg·mL^{-1} was used to set up co-crystallization with antimicrobial agents with concentration of 100 times of the protein concentration. After the protein and antibiotic were incubated for one hour at 25 °C, the crystallization was performed using hanging drop method in 24-well plates with two drops of different volume ratios of protein to precipitant, 1.0:1.0 and 1.0:1.5 (μL:μL) were set up on an 18-mm cover slide. The condition G8 (33% PEG400, 0.1 M sodium chloride, 0.1 M MES, pH 6.5) from MemGold1™ and the condition E10 (33% PEG400, 0.23 M

sodium chloride, 0.05 M sodium acetate, pH 4.5) from MemGold2™ screening kits (Molecular dimension, Germany) were optimized the precipitant concentrations, and PEG400 was varied from 28% - 33%. The plate was incubated at 19 °C and observed daily under a stereo microscope. In addition, the complex crystals were set up by using another technique which is soaking. Apo crystals were set up with the same method. After the crystal appeared, 1 μL of antibiotic with a concentration of 1 M was added into the hanging drop on cover slide the in 24-well plates and continued to incubate for 24 h. Single crystals obtained from the optimized condition were harvested in a nitrogen cryo-stream using 20% (v/v) glycerol as a cryoprotectant, and further tested for X-ray diffraction.

2.5 Molecular docking

The antibiotic-*Ec*ChiP structural prediction was carried out by molecular docking, using the unpublished complex crystal structure of *Ec*ChiP with $(GlcNAc)_6$ as the structural template. The 3D structures of antibiotics were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Initially, all water molecules, ions and ligands were removed from the crystal structure, and the other parameters were set up by default. The automatic genetic algorithm (GA) parameter setting was used for the GOLD docking calculations (12). In order to define the ligand binding site, only amino acid residues of *Ec*ChiP within a 4 Å radius from the $(GlcNAc)_6$ were considered. The number of GA runs was set to 150. The results were shown as docking scores available in GOLD. The highest score from GOLD rank was selected to be the suitable conformer for antibiotics inside the *Ec*ChiP channel.

3. Results

3.1. Antimicrobial susceptibility of E. coli DH5α

To study antimicrobial susceptibility of *E. coli* DH5α, two methods were reported in this work; 1) Microdilution Inhibitory assay and 2) agar disc diffusion assay. Results obtained from both methods were evaluated in comparison with the breakpoints set by the European Committee on Antimicrobial Susceptibility Testing EUCAST (http://www.eucast.org/). The antimicrobial susceptibilities under disc agar assay showed that almost all antibiotics (imipenem, meropenem, doripenem, minocycline, doxycycline, co-trimoxazole, sulfamethoxazole, trimethoprim, ciprofloxacin, norfloxacin, kanamycin, amikacin, gentamicin, tobramycin, ampicillin, amoxicillin, chloramphenicol, tigecycline, cefotaxime, cefoxitin, cefepime, and ceftazidime) could inhibit the bacterial growth from lowest to highest amount of the antibiotics used, except dicloxacillin, as observed from the size of clear zones that developed around the antibiotic-immersed dises placed on the bacterial grown plates (Figure 1). The diameters of the clear zones were measured and shown in Table 1. The increased amount of each antibiotic did not significantly increase the size of the clearing zone. The diameters of the clear zone at 10, 20, 30 and 40 μg disc contents are the same. When comparing the results of this experiment with EUCAST, the results are relatively close to the EUCAS experiments, but the clear zone sizes obtained from this experiment were rather larger when measured at the same amount of the antibiotic in disc content. Table 2 presents the MIC values obtained from this study comparing with the standard values from EUCAST. The result shows that among eight antibiotics tested, *E. coli* was shown to be subjectible towards three antibiotics including co-trimoxazole, trimethoprim, and chloramphenicol. Note that there are no values reported for dicloxacillin, minocycline, doxycycline, and sulfamethoxazole from EUCAST, therefore the susceptibility/resistance level of the studied *E. coli* DH5α strain cannot be evaluated for these antibiotics. After that, the susceptible antibiotics were used for a study about the permeation of antibiotics through *Ec*ChiP by using BLM technique.

Figure 1. Agar discs assay of antibiotics against *E. coli* DH5α. The agar plate was incubated at 37 °C for 16 hours with the disc content about 0, 10, 20, 30 µg.

Table 1. Resistance zone diameter for *E. coli* against different antibiotics compar with the standard values from EUCAST

Class	Antibiotics	Clear zone of *E. coli* DH5α (mm)				EUCAST zone inhibitory (mm) (disk contents)	
		10 µg	20 µg	30 µg	40 µg	S ≥	R <
Carbapenem	Imipenem	11	11	11	12	22 (10 µg)	19 (10 µg)
	Meropenem	29	33	34	36	22 (10 µg)	16 (10 µg)
	Doripenem	31	36	36	37	24 (10 µg)	21 (10 µg)
Tetracycline	Minocycline	19	22	26	26	-	-
	Doxycycline	20	21	21	23	-	-
Co-trimoxazole	Co-trimoxazole	34	34	36	37	14 (25 µg)	11 (25 µg)
	Sulfamethoxazole	23	25	30	34	-	-
	Trimethoprim	35	39	40	42	15 (5 µg)	15 (5 µg)
Fluoroquinolone	Ciprofloxacin	41	41	41	41	22 (5 µg)	22 (5 µg)
	Norfloxacin	38	40	40	41	22 (10 µg)	22 (10 µg)
Aminoglycoside	Kanamycin	27	28	30	31	-	-
	Amikacin	27	30	31	32	18 (30 µg)	18 (30 µg)
	Gentamicin	31	31	36	36	17 (10 µg)	17 (10 µg)
	Tobramycin	30	34	35	37	16 (10 µg)	16 (10 µg)
Penicillin	Ampicillin	11	19	23	24	14 (10 µg)	14 (10 µg)
	Amoxicillin	6	18	18	19	-	-
Chloramphenicol	Chloramphenicol	26	36	41	41	17 (30 µg)	17 (30 µg)
Glycylcycline	Tigecycline	8	10	11	12	18 (15 µg)	18 (15 µg)
Cephalosporin	Cefotaxime	30	31	34	36	20 (5 µg)	20 (5 µg)
	Cefoxitin	21	26	29	30	19 (30 µg)	19 (30 µg)
	Cefepime	32	33	35	36	27 (30 µg)	24 (30 µg)
	Ceftazidime	26	28	30	31	22 (10 µg)	19 (10 µg)

Table 2. MICs values for *E. coli* against antibiotics compared with the standard values from EUCAST

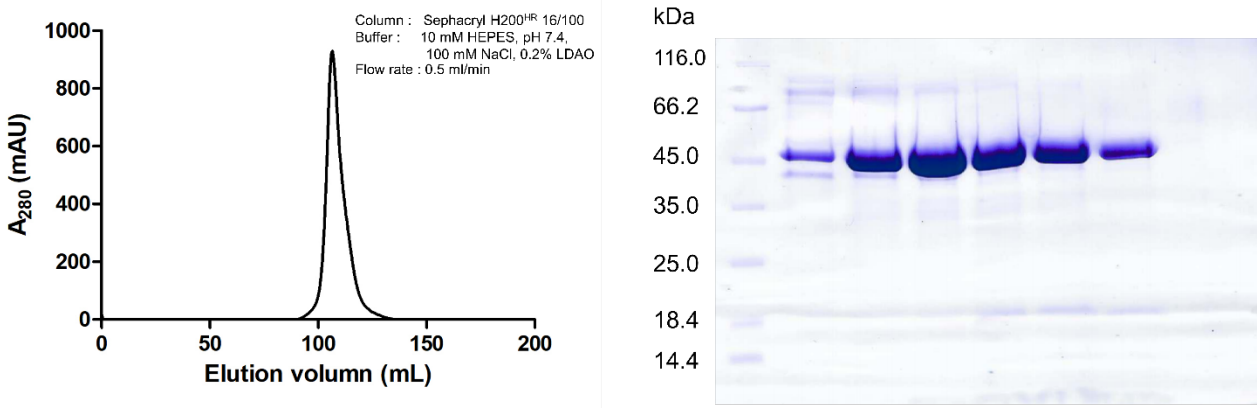
Antibiotics		EUCAST MIC ($mg \cdot mL^{-1}$)[1]		MIC Value ($mg \cdot mL^{-1}$)
		S≤	R>	
Penicillin	Dicloxacillin	-	-	2048
Tetracycline	Minocycline	-	-	4
	Doxycycline	-	-	1
Glycylcycline	Tigecycline	0.5	0.5	-
Co-trimoxazole	Co-trimoxazole	2	4	1
	Sulfamethoxazole	-	-	1024
	Trimethoprim	4	4	2
Chloramphenicol	Chloramphenicol	8	8	4

[1]The values presented are obtained from the experiments performed 4 times. [a]Breakpoints defined for *E. coli.* follow the EUCAST Clinical Breakpoint. R, Resistant; S, Susceptibility; -, No breakpoints.

*3.2. Expression and purification of the recombinant Ec*ChiP

The recombinant *Ec*ChiP was successfully expressed in *E. coli* BL21(DE3) Omp8 Rosetta strain. After cell lysis, the *Ec*ChiP containing peptidoglycan fraction was prepared by extraction with 2% (w/v) SDS followed by 2% (v/v) LDAO. After extraction step, the protein was purified by ion-exchange chromatography and furtherly purified to homogeneity by size exclusion chromatography using Sephacryl $H200^{HR}$ 16/100 column chromatography. Figure 2a is a representative elution profile of *Ec*ChiP, showing a single A_{280} peak containing a major protein band (stained with Coomassie blue) that migrated with an apparent MW of approximately 45 ~ 50 kDa on SDS-PAGE (Fig. 2b). The size of the protein observed on SDS-PAGE gel corresponded to the size of *Ec*ChiP reported

previously (2, 10). After that, the highly purified *Ec*ChiP fractions were pooled together, concentrated to 10 mg·mL^{-1} and used for crystallization and black lipid membrane (BLM) experiments.



(**a**) (**b**)

Figure 2. Preparation of *Ec*ChiP expressed in *E. coli* BL21(DE3) Omp8 Rosetta strain: (a) A representative elution profile of *Ec*ChiP from a HiPrep 16/100 Sephacryl S-200^{HR} column eluted with 10 mM HEPES, pH 7.4, 100 mM NaCl, 0.2% LDAO; (b) SDS-PAGE showing that a single protein peak with an elution volume of 100 to 120 mL (fractions F34-F40).

3.3. Permeation of antibiotics through EcChiP by BLM

According to the results of MIC and agar disc diffusion assays, the susceptible antibiotics were chosen for the single-channel study of antibiotic interaction to *Ec*ChiP. Figure 3 shows typical ion traces of *Ec*ChiP exposed to different antibiotics (2 mM of each antibiotic was added to the *cis* side) at the potential adjusted to ±100 mV. The fluctuation of the current traces can be used to identify the interaction between antibiotics and *Ec*ChiP. At ±100 mV, minocycline and tigecycline were found to occlude the channel but minocycline generated a more strongly signal than at -100 mV than +100 mV. In contrast, gentamycin interacted with the channel only at +100 mV, while no interaction was observed at -100 mV. The results suggested that the interactons of EcChiP and charged antibiotics were asymmetric, depending on the net charge(s) on their molecules. For the rest of the drugs, no interaction between the antibiotics and *Ec*ChiP at both negative and positive potentials.

3.4. Co-crystallization of EcChiP with antibiotics

From BLM experiments, the antibiotics which can interact with *Ec*ChiP channel are gentamycin, minocycline and tigecycline (Figure 3). These antibiotics were used for co-crystallization with *Ec*ChiP. Single crystals of *Ec*ChiP co-crystallized with minocycline and tigecycline were observed after 10 days. The crystals were grown in the condition E10 (33% PEG400, 0.23 M sodium chloride, 0.05 M sodium acetate, pH 4.5) from MemGold**2**™ and single crystals from soaking with minocycline, tigecycline and gentamycin were grown in the condition G8 (33% PEG400, 0.1 M sodium chloride, 0.1 M MES, pH 6.5) from MemGold**1**™. The crystals have a long tetragonal with approximate dimensions of around 300 – 500 μm in both conditions. For other antibiotics including gentamycin and tigecycline, no crystals appeared.

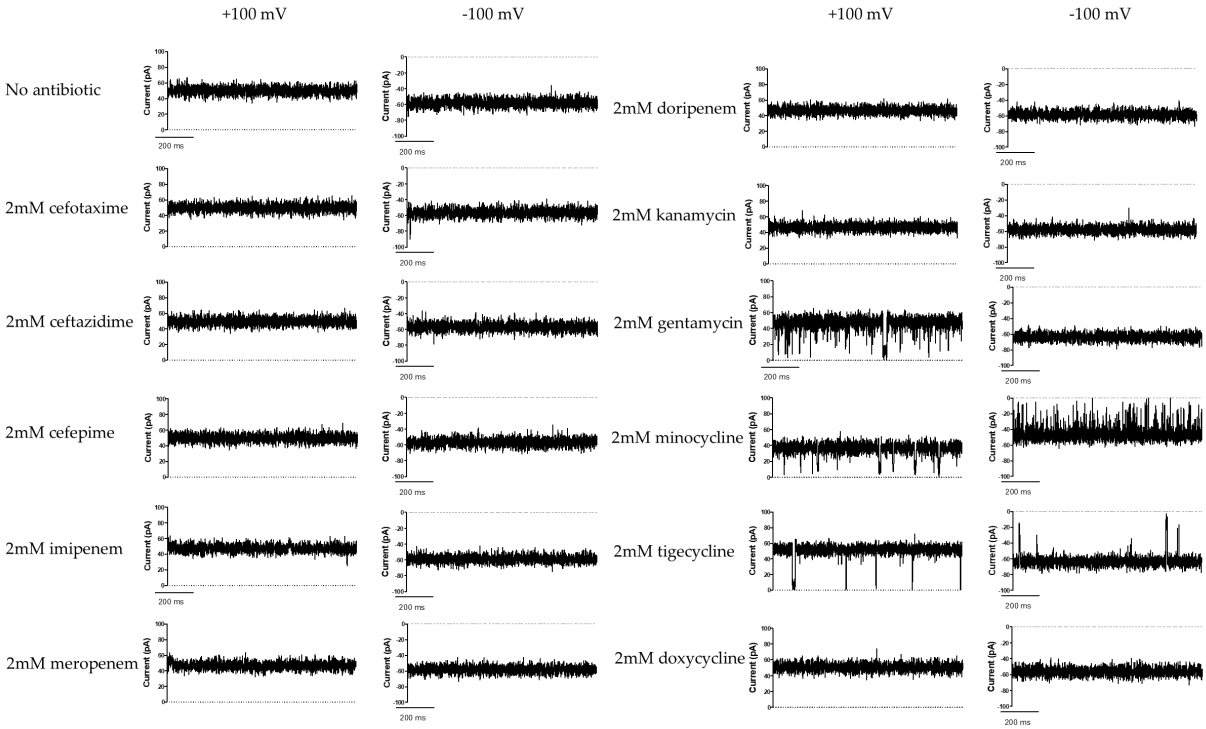


Figure 3. Ion traces of *Ec*ChiP exposed to 2 mM of different types of antibiotics. The data were acquired at ±100 mV, with each antibiotic was added on the *cis* side.

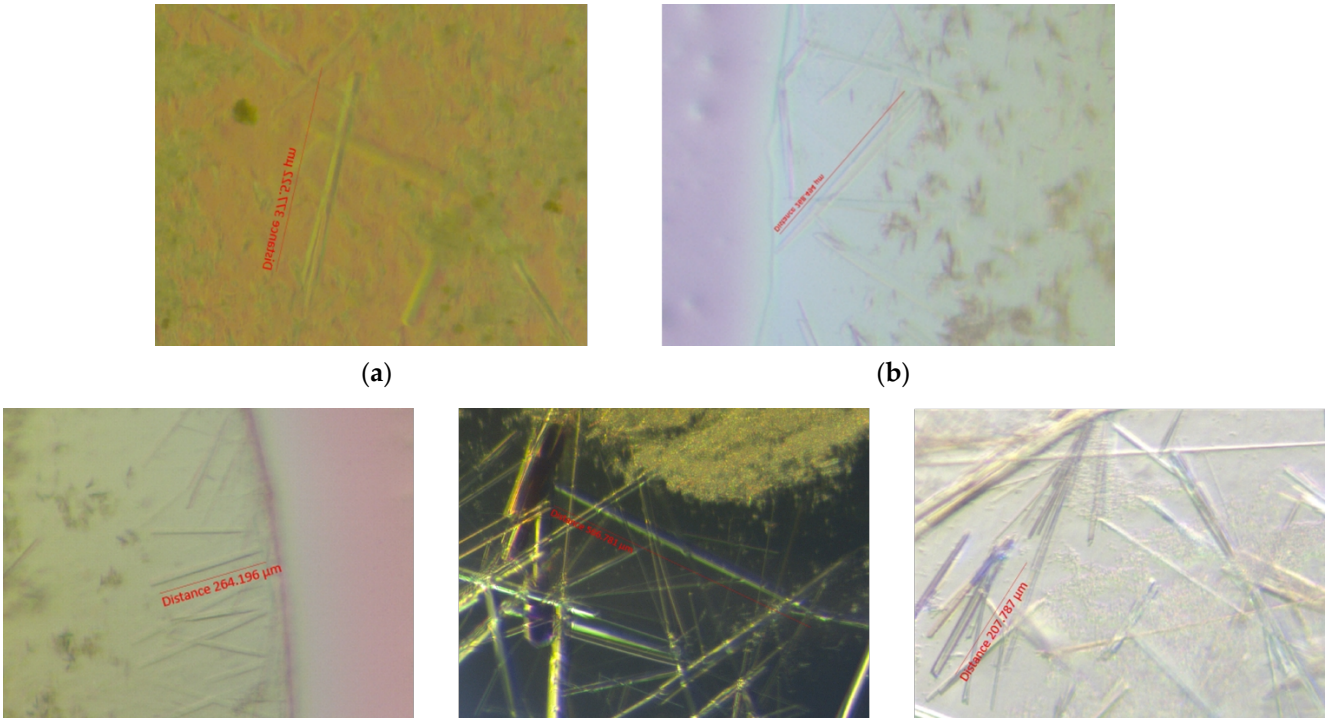


Figure 4. Single crystals of *Ec*ChiP co-crystallized in the condition E10 (33% PEG400, 0.23 M sodium chloride, 0.05 M sodium acetate, pH 4.5) from MemGold2™; (a) minocycline and (b) tigecycline. Single crystals from soaking were observed in the condition G8 (33% PEG400, 0.1 M sodium chloride, 0.1 M MES, pH 6.5) from MemGold1™; (d) minocycline, (e) tigecycline, and (f) gentamycin.

3.5. Antibiotics-EcChiP structural prediction by molecular docking

According to the results of single-channel experiments, *Ec*ChiP can interact with gentamycin, minocycline and tigecycline. Since we did not obtain the structure complexes of EcChiP with All structures were predicted by molecular docking using GOLD program(12). Figure 5 shows the predicted structures with the antibiotic inside the channel. After that, The antibiotic-channel interactions were analyzed by LIGPLOT(17). Figures 5(a), (d) and (g) show the interactions of *Ec*ChiP and minocycline. As seen, *Ec*ChiP uses the residues; Aln^{294}, Arg^{314}, and Asp^{316} to make hydrogen bonds and uses the residues; Glu^{78}, Trp^{132} and Trp^{310} to make hydrophobic interactions with minocycline. Figures 5(b), (e) and (h) are the interactions of *Ec*ChiP and tigecycline, showing His^{131}, and Arg^{295} as the key binding residues to make hydrogen bonds and the residues; Trp^{132}, Tyr^{300} and Trp^{310} to make hydrophobic interactions with tigecycline.The interactions of *Ec*ChiP and gentamycin are analyzed, Gentamycin occupies the construction zone of the *Ec*ChiP channel by forming hydrogen bonds with Arg^{18}, Trp^{132}, Ser^{133}, Asp^{380}, and Lys^{426} and hydrophobic interactions with the residues; Trp^{16}, Trp^{310}, Arg^{314}, Val^{382}, Lys^{396}, His^{398}, and Met^{428}.

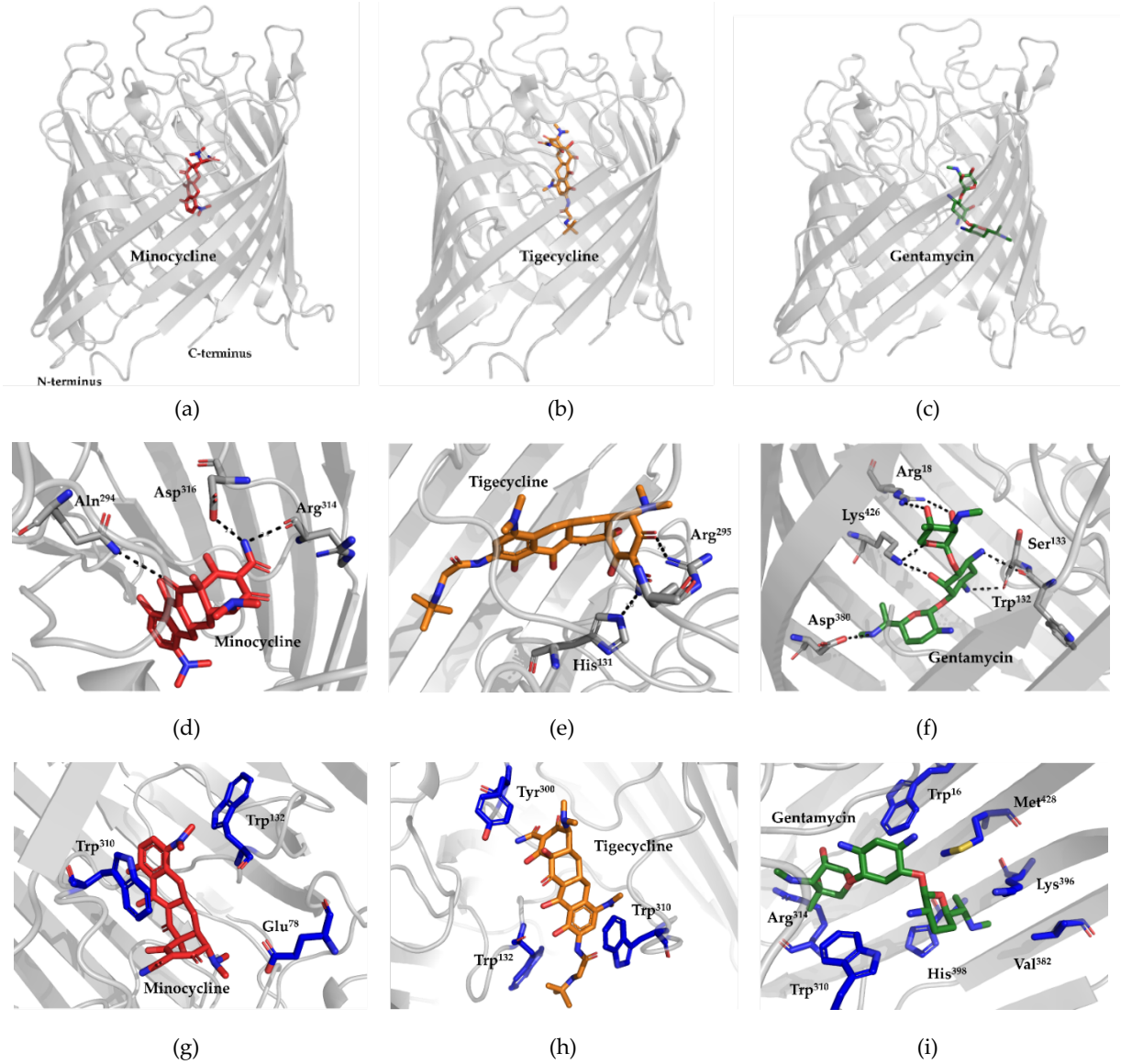


Figure 5. Prediction of the structure of *Ec*ChiP in complex with antibiotics; (a) minocycline, (b) gentamycin, and (c) tigecycline. All structures were analyzed for specific interactions by LIGPLOT program; Hydrogen bond of (d) minocycline, (e) gentamycin, and (f) tigecycline. Hydrophobic bond of (g) minocycline, (h) gentamycin, and (i) tigecycline.

4. Discussion

EcChiP was identified to be responsible for the uptake of chitooligosaccharides. Its biological functions were characterized by using electrophysiological and biochemical approaches (2, 10). Previous reports from single-channel analysis demonstrated that EcChiP is a stable monomeric channel in artificial phospholipid membranes, with an average conductance of 0.55 ± 0.01 nS. EcChiP channels showed specific interactions to long-chain chitooligosaccharides (2, 10). In conjunction with liposome swelling assay, which shows that EcChiP also allowed small molecules with the limited sizes of 200-300 Da to permeate through EcChiP by general diffusion and long -chain chitooligosaccharides were passed through by facilitated diffusion (2, 10). It was also reported that the antibiotics could pass through outer membrane porins by general diffusion as well (18). This study demonstrates that antimicrobial agents permeated through a sugar-specific channel and caused cell death. The antibiotics including gentamycin, minocycline and tigecycline are able to inhibit the E.coli growth, while the accepted MICs values are not presenting in the EUCAST table. The current traces obtained from the single-channel experiments indicate that three antibiotics (gentamycin, minocycline and tigecycline) can interact with the internal residues of EcChiP. However, to confirm the permeability of these antibiotics through the channel, further single-channel experiments should be constructed, the concentration titration of antibiotics should be performed.

The crystal structures of EcChiP in complex with the active antibiotics may explain the molecular basis of the transport event. Currently, the crystals of EcChiP co-crystallized with minocycline were just obtained, but the X-ray diffraction data are still to be collected and analyzed. For other antibiotics, further optimizations have to be done. Molecular docking is another tool for structure prediction, here, we present the complex structure prediction of EcChiP with gentamycin, minocycline and tigecycline. The interactions show that EcChiP uses amino acid residues such as Arg314, Ser133 to make hydrogen bonds with the antibiotic molecues, and Trp310 locates at the constriction zone is the key residues that make hydrophobic interaction with the modeled antibiotics. This residue is also important for the (GlcNAc)6 – EcChiP interactions.

5. Conclusions

The results of antibiotics susceptibility against E. coli show that most antibiotics were effective against E. coli DH5◎ strain, consistent to the standardised values reported from EUCAST. After that, the recombinant EcChiP was successfully expressed in E. coli BL21(DE3) Omp8 Rosetta strain and can be used for crystallization and single-channel experiments. According to the results of MIC and agar disc diffusion assay, the study of the permeation of antibiotics through EcChiP was elucidated by BLM experiments. Screening for ion traces of EcChiP in the presence of antibiotics indicated that gentamycin, minocycline and tigecycline could interact with EcChiP channel. These antibiotics were used as the ligands for co-crystallization with EcChiP in order to further study the structure of the EcChiP that interacts with antibiotics. However, we only found the co-crystals of EcChiP and minocycline. Besides that, we try to predict the structure of EcChiP and antibiotics by molecular docking via the GOLD program (12) using unpublished data of EcChiP in complex with (GlcNAc)6 as the structural template. Finally, we have received the predicted structure of EcChiP complex with minocycline, gentamycin and tigecycline. All structures were analyzed via LIGPLOT program (17), the interactions between EcChiP and antibiotics indicate that minocycline, gentamycin and tigecycline have ability to pass through the EcChiP pore. In conclusion, understanding the antibiotics-EcChiP interactions may suggest an effective approach to design novel anti-microbial agents against the infectious strains of Escherichia coli.

Supplementary Materials: All materials are contained within this article.

Author Contributions: P.B. conducted the experiments, generated, and analyzed the data, wrote the first manuscript draft, and prepared all the figures. A.A. studied BLM experiment. W.S. guided and revised the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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