Role of chitooligosaccharide-specific channel in aminoglycoside uptake by the opportunistic pathogen *Serratia marcescens*

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Abstract: *Serratia marcescens* (*Sm*) is an opportunistic pathogen that is highly infectious to the immunocompromised patients. There are several reports of high antibiotic resistance rate against the first generation of β-lactam and cephalosporin antibiotics. The clinical practice report establishes that the use of aminoglycosides in combination with other types of antibiotics can decrease the resistance rate of this bacterium. Recent studies of chitin utilization in *Sm* showed that *Serratia spp.* can uptake chitin nutrients through a sugar-specific porin named chitoporin (*Sm*ChiP). All aminoglycosides contain three sugar rings in their backbone structures with different side chains. Therefore, the molecular uptake of these antibiotics is thought to be able to pass through sugar-specific porins, such as chitoporin. In this work, the aminoglycoside susceptibility of *Sm* was carried out based on the EUCAST standardize protocol. The results show that only gentamicin is in the susceptible range of MICs values (2 mg.mL^{-1}). Single channel recording experiments show that only gentamicin and kanamycin B strongly interact with the *Sm*ChiP channel. The fluctuation of current was observed only at the negative potential applied because of the positively-charged amine groups of gentamicin and kanamycin B. Docking gentamicin and kanamycin B into the pore interior of *Sm*ChiP showed that both antibiotics occupied the same constriction area with the known substrate, chitohexaose. Understanding antibiotics uptake through *Sm*ChiP can help to design new effective anti-microbial drugs against *Sm*.

Keywords: *Serratia marcescens*, Chitoporin, Aminoglycosides, Gentamicin, Kanamycin, Black Lipid Membrane (BLM), Molecular Docking

1. Introduction

Serratia marcescens (*Sm*) is one of the species in Gram-negative bacteria that belongs to the family *Enterobacteriaceae* [1,2]. It has a widespread existence in different environments, such as soil, water, plants and the digestive tracts of animals and humans. *Sm* is an opportunistic pathogen that commonly infects urinary tract, bloodstream and respiratory tract and gastrointestinal systems of hospitalized persons [1–3]. Nowadays, the uses of a combination of different types of antibiotics have been suggested for effective treatment of the *Sm* infection. Numerous reports showed that *Sm* develops antibiotic resistance against cephalosporins and β-lactam antibiotics [4,5]. According to the up-to-date clinical protocol for *Sm* treatment, the uses of aminoglycosides are more successful, and help to reduce the resistance rate of *Sm* than other types of antibiotics [6,7].

Small molecules usually enter the periplasm of the Gram-negative bacteria through the outer membrane protein transporters, such as porins. The studies of normal diffusion porins for the antibiotic transport have been revealed in the recent years [8]. Molecular simulations and electrophysiological studies showed that OmpF and OmpC could

interact with kanamycin, one of the aminoglycosides, and the drug was suggested to be translocated by these porins. However, the solute-specific porins have no report to be responsible for antibiotic uptake. Chitoporin is one of the specific porins, which had been discovered and its specific function on chitooligosaccharide, especially chitohexaose ((GlcNAc)$_6$) uptake was elucidated recently [9–12]. The studies of chitoporin from *Sm,* so-called *Sm*ChiP, have now been reported. The OM-expressed *Sm*ChiP was successfully expressed and purified to homogeneity. Crystallization of *Sm*ChiP in complex with (GlcNAc)$_6$ yielded single crystals, and one of which diffracted the X-ray light source at the 2.7 Å resolution [13]. In this work, we investigated the aminoglycoside susceptibility of *Sm* strain ATCC® 14756. Interactions of *Sm*ChiP in lipid membranes with aminoglycosides, such as amikacin, kanamycin B, gentamicin, tobramycin, and isepamicin are also demonstrated.

2. Materials and Methods

2.1. Microdilution assay

Susceptibility of *Sm* to aminoglycosides including amikacin, tobramycin, kanamycin, isepamicin, and gentamicin were determined by agar disc and microdilution assays [14,15]. *Sm* type strain ATCC® number 14756 (American Type Culture Collection, Manassas, VA, USA) was used in this work. Although the MIC values and zone diameters were compared to the standardized values reported in the EUCAST Table 2020 (The European Committee on Antimicrobial Susceptibility Testing, with the breakpoint tables for interpretation of MICs and zone diameters, version 10.0, 2020: http://www.eucast.org/clinical_breakpoints/). Cells were grown in the Muller-Hilton (MH) medium. until $OD_{600} \approx 0.6$, then the cells were transferred to a sterile 96 well-plate and diluted with sterilized normal saline (0.9% of NaCl) to $OD_{600} \approx 0.1$. Then, 200 μL of the MH medium containing a serial dilution of antibiotics (a concentration range of 0.0158 to 2048 mg.mL^{-1}) were added into the 96 well-plate. Next, 10 μL of cell solution was transferred into the microtiter plate. Cell growth and MICs values were evaluated after 16 hours of incubations at 26 °C using a microplate reader (Thermo Fisher Scientific, Finland) at OD_{600}. The MICs values were presented at the concentration of antibiotics that could completely inhibit the cell growth.

2.2. Recombinant expression and purification

Recombinant plasmid in vector pET23a(+)/*Sm*ChiP was transformed to the omp deficient *E. coli* BL21 (DE3) Omp8 rosetta strain, then transferred to Luria-Bertani (LB) broth containing 100 μg.mL^{-1} ampicillin and 25 μg.mL^{-1} kanamycin and grown at 37 °C at 180 rpm. *Sm*ChiP expression was induced with 0.5 mM (final concentration) isopropyl thio-β-D-galactoside (IPTG) for 6 hours. Cells were harvested by centrifuge at 8000 rpm, 4 °C for 15 min. After that, cells were re-suspended in lysis buffer contained 20 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$ 0.1 mM $CaCl_2$ with 0.1% (v/v) TritonX-100, then, solution was disrupted on ice with a high-speed ultrasonic processor (Cole-Parmer, Vernon hills, Illinois, USA) for 10 min, after that, the 2% (w/v) SDS was added and incubated at 50 °C for 60 min with 300 rpm using thermomixer comfort (Eppendorf AG, Hamburg, Germany) with thermoblock (4 x 50 mL) Falcon tubes. Cell wall components were removed by ultracentrifugation at 22,000 rpm at 4 °C for 45 min. The pellets with *Sm*ChiP were extracted twice by 2.5% Octyl-POE in 20 mM phosphate buffer pH 7.4, the supernatant was collected by ultracentrifugation at 40,000 rpm 25°C for 1 hour. *Sm*ChiP was purified by size exclusion chromatography using HiPrep 26/100 Sephacryl S-200 high-resolution column to ÄKTA™ pure FPLC (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), with a flow rate of 1.0 mL.min^{-1}. The eluted fractions were checked purity by SDS-PAGE. The purified fractions were pooled and determined the concentrations by A_{280} with the molar extinction coefficient of 12,6170 M^{-1}cm^{-1}.

2.3. Single channel studies

Black lipid membrane (BLM) measurements were carried out at room temperature using electrolyte containing 1 M KCl in 20 mM phosphate buffer pH 7.4. Phospholipid was prepared using 5 mg.mL^{-1} of 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC; Avanti Polar Lipids, Alabaster, AL) in *n*-pentane. A Teflon film with an aperture of 50-100 μm was sandwiched with two chambers and pre-treated the aperture by a few microliters of 1% (v/v) hexadecane in hexane, then lipid bilayer was formed between two chambers by lowering-rising technique [16]. Ion current was measured by Ag/AgCl electrodes, one was connected to the ground (*cis*) and the other one to the Axon 1550® amplifier (Molecular Device, Germany) (*trans*). The trades were collected at 10 kHz sampling rate with low pass Bassel (8-pole) filter. A few microliters of *Sm*ChiP from a stock of 2 mg.mL^{-1} was always added to the *cis* side of the cuvette. To investigate the effects of the aminoglycoside antibiotics, once a stable *Sm*ChiP channel was inserted in the lipid membrane, 1 mM of each antibiotic was added on the cis side of the chamber, and its interactions with the channel were observed. The data were acquired at ±100 mV, for 2 min.

2.4. Molecular docking

The crystallographic data for *Sm*ChiP complex with chitohexaose was obtained from the unpublished data with the 2.7 Å resolution. Molecular docking was initiated by introducing hydrogen atoms into the *Sm*ChiP molecule using the ionization and tautomeric states inferred by the GOLD program [17], while all water molecules, ions and ligands were removed from the crystal structure. In the docking procedure, other parameters were adjusted by default. The automatic genetic algorithm (GA) parameter setting was used in all the GOLD docking calculations with the 150 possible protein-liganded conformers iteration. To define the key sugar binding sites, only amino acid residues of *Sm*ChiP within a 6 Å radius from the center of the bound chitohexaose were considered. Docking trials were carried out by adding gentamicin and kanamycin B, which the structures were obtained from PubChem (Gentamicin; CID72395, Kanamycin B; CID25245850). The scoring function used in all docking calculations was 'ChemPLP', available in GOLD v.5.3 [18,19]. All the GOLD docking calculations and were further investigated for protein-ligand interactions. The conformer with the highest ChemPLP fitness docking score was selected for protein ligand analysis. The results were visualized by PyMOL [20].

3. Results

3.1. Microdilution and disc agar assays

There are two methods reported in this work to determine antibiotic susceptibility of *Sm*: microdilution broth assay and antibiotic disc agar assay. **Table 1** presents the statistical value of the MIC values obtained from this study comparing with the standard values from EUCAST. For unknown standard data, the results were reported as observed data for this *Sm* strain. In addition, under disc agar assay, the results show that most of the tested aminoglycoside can kill the bacterial cells, as observed from the size of clear zone (**Figure 1**) and resistance zone diameters are given in **Table 1**. From all aminoglycosides, gentamicin was most active, with an MIC value of 2 mg.mL^{-1} and its effect on the *Sm* growth was confirmed by disc agar assay that showed a large clear zone with a diameter of 19 mm at the lowest amount (10 μg) of the antibiotic, and the clear zone become larger to 22 mm and 24 mm, when the amount of the antibiotic was increased to 20 μg, and 30 μg, respectively. Other antibiotics (amikacin, isepamicin, tobramycin, kanamycin A, kanamycin) also inhibited the growth of *Sm*, but greater amounts (20 and 30 μg) were required to see the visible effects (**Figure 1**)

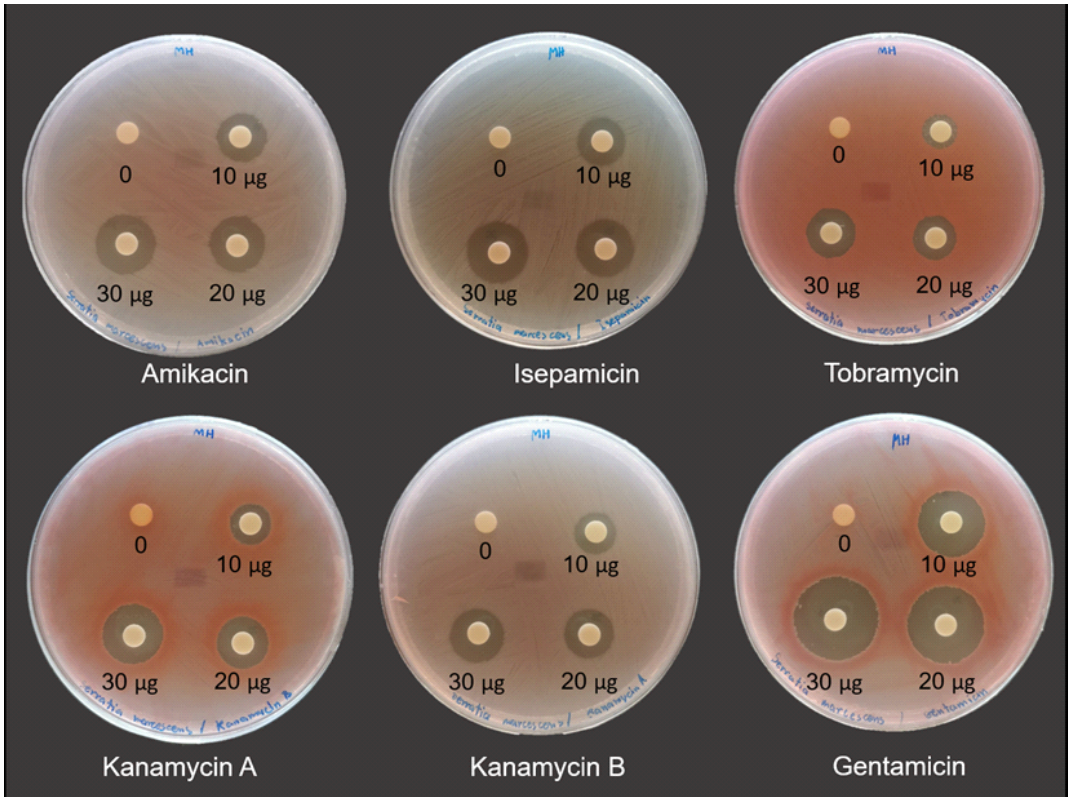

Figure 1. Agar discs assay of different aminoglycosides against *S. marcescens*. The agar plate was incubated at 26 °C for 16 h. Aminoglycosides include amikacin, isepamicin, tobramycin, kanamycin A, kanamycin B, and gentamicin and with the disc contents of 10, 20, 30 µg.

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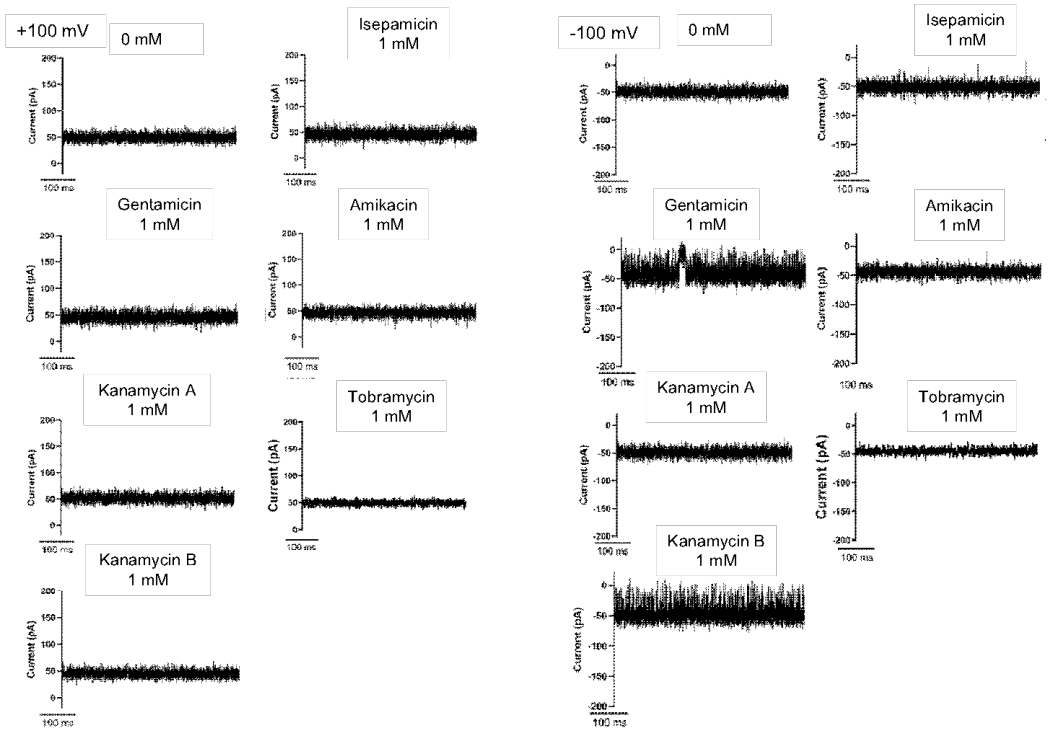
Antibiotics	10 µg (mm)	20 µg (mm)	30 µg (mm)	EUCAST zone inhibitory (mm) (disk contents)		MIC (mg.mL^{-1})	EUCAST MIC (mg.mL^{-1})	
				S ≥	R <		S ≤	R >
Amikacin	14	15	16	18 (30 µg)	18 (30 µg)	16	8	8
Tobramycin	10	12	14	17 (10 µg)	17 (10 µg)	8	2	2
Gentamicin	19	22	24	17 (10 µg)	17 (10 µg)	2	2	2
Isepamicin[1]	13	15	16	-[-]		-	-[-]	
Kanamycin A[1]	12	13.5	15	-[-]		8	-[-]	
Kanamycin B[1]	11	14	16	-[-]		8	-[-]	

[1] no data reported in EUCAST 2020 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf)



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

Figure 2. Ion traces of *Sm*ChiP exposed to 1 mM of aminoglycosides which were added on the cis side. The data acquired at (**a**) +100 mV, (**b**) at – 100 mV.

3.2. Single-channel studies of the antibiotic and sugar-analog interactions with SmChiP

The interactions between single channel of *Sm*ChiP and the aminoglycosides were investigated using black lipid membrane (BLM) reconstitution technique. **Figure 2** shows ion flowthrough *Sm*ChiP in the absence and presence of different aminoglycosides (1 mM of each compound was added at the *cis* side) with the potential ±100 mV. The tested antibiotics included gentamicin, kanamycin A and B, isepamicin, amikacin, and tobramycin. It is shown that at 1 mM only gentamycin and kanamycin B interrupted the ion flow in the negative potential, causing transient drops of the current (**Figure 2b**). On the other hand, other antibiotics did not visibly affect the ion flow. More transient blocking events were seen with kanamycin B. The observed ion fluctuation indicated that the drug molecules occluded, and then interacted with the *Sm*ChiP channel.

3.3. Molecular docking

According to the results obtained from single-channel experiments, *Sm*ChiP can interact with gentamycin and kanamycin B (**Figure 2**). Consistent with single-channel analsis, molecular docking of gentamicin and kanmycin B into *Sm*ChiP pore using GOLD program showed the ChemPLP score of 60.13 for gentamicin and 66.85 for kanamycin B. Both antibiotics were modelled inside the protein pore (**Figure 3a-b**). Superimposition of the modelled antibitobics inside the protein pore, with the chitooligsaccharide substrate, chitohexaose showed that the locations of both antibiotics do not completely align with the structure of chitohexaose, in the structural complex with *Sm*ChiP-chitohexaose (**Figure 4a**). These results show that kanamycin B and gentamicin adopted different oreintations inside the channel but the sugar ring (ring C in **Fig. 3c**) of the two anitbiotics occupied the central part of the affinity site and in the same position ofwith $(GlcNAc)_3$ of chitoheaxoseas shown in **Figure 4**. The interactions of *Sm*ChiP and the two aminoglycosides were analyzed by LIGPLOT with the distance between the donor and the aceptor atom of hydrogen bond was set at 3.5 Å and hydrophobic interaction at 4.0 Å. The side chains of Glu^{82} Ser^{137} Asp^{320}

and Lys^{425}, and the main chain of Trp^{136} and Arg^{318} are found to form hydrogen bonds with the three sugar moieties of the gentamycin molecule. Hydrophobic interactions are observed between the side chain of Ile^{83} and especially the aromatic ring of Trp^{314} and Trp^{16} stack against the facets of the sugar ring B and C (**Figure 3d**). The interactions of the channel and kanamycin B are presented in **Figure 3e**. The hydrogen bond network is made up by Ser^{137} Arg^{18} Glu^{82} Arg^{318} Asp^{320} Asn^{416} Lys^{425} Arg^{299} and His^{135}, and the hydrophobic interaction comprises Trp^{16} and Tyr^{304} stacking with ring C and ring A, respectively. The main interactions are found in the same area with GlcNAc-2 to GlcNAc-4 of chitohexaose which comprised of amino acid residues include Ser^{137} (GlcNAc-2) Arg^{18} (GlcNAc-3) Glu^{82} (GlcNAc-3) Asp^{320} (GlcNAc-4) Asn^{416} (GlcNAc-4) for hydorgen bond, and Tyr^{304} (GlcNAc-4) Trp^{314} (GlcNAc-2) and Trp^{16} (GlcNAc-2) for hydrophobic interactions (**Figure 4b**).

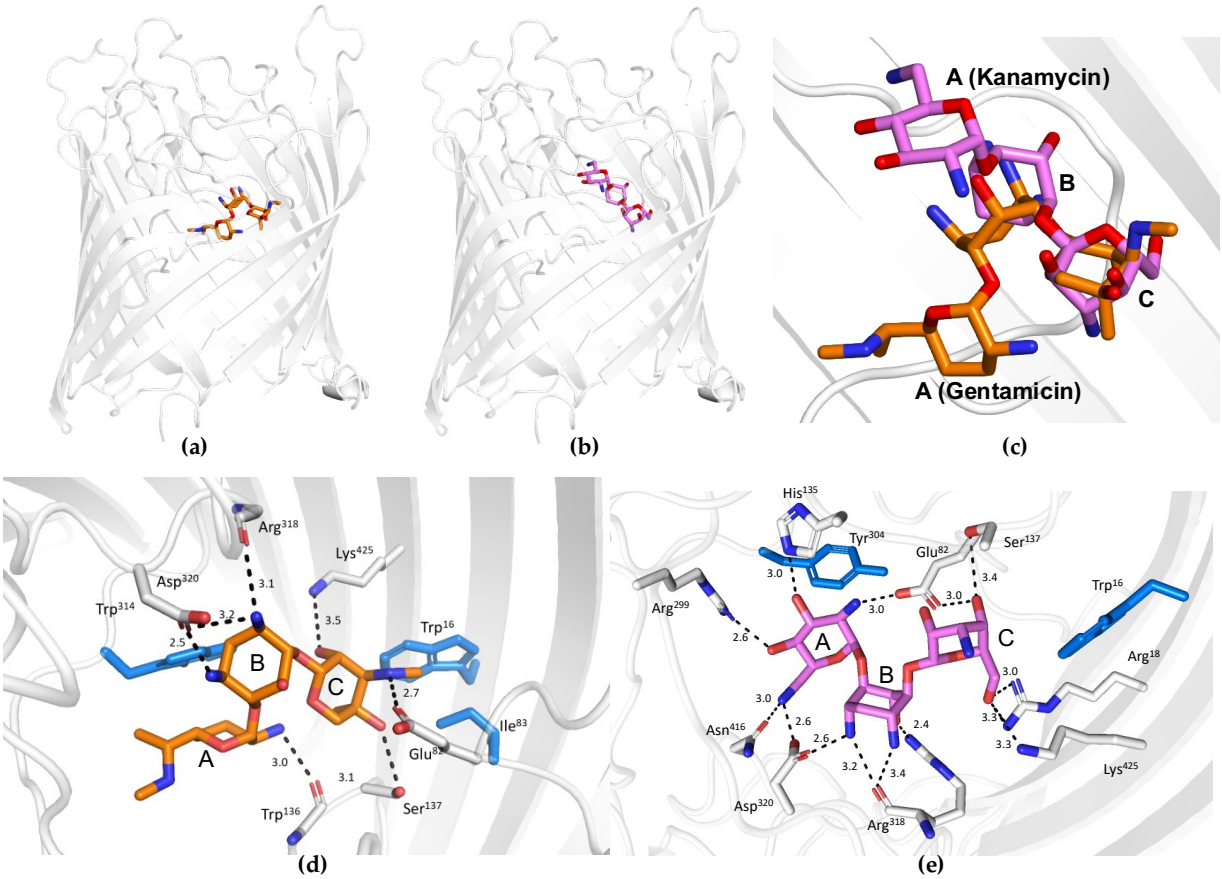


Figure 3. Molecular docking visualization of the highest fitness score from GOLD. Protein was presented in cartoon representative (white color) and antibiotics were presented as stick. (a) Side view of *Sm*ChiP with gentamicin, which is presented in orange color. (b) Side view of *Sm*ChiP (white) and kanamycin B (hot pink). (c) The superimposition of gentamicin and kanamycin B, showing aminocyclitol, ring B, in the structure are faced in the same area, whereas ring B presented the opposite orientation with each other caused ring A showing in the different area. (d) The interaction of *Sm*ChiP with gentamicin (stick with orange color) comprised by hydrogen bond (white) and hydrophobic (blue) interaction. Hydrogen bond network of *Sm*ChiP and gentamicin are the side chains of Glu^{82} Ser^{137} Asp^{320} and Lys^{425}, and the main chain of Trp^{136} and Arg^{318}, and hydrophobic interaction made by Ile^{83} Trp^{314} and Trp^{16}. (e) The interaction of *Sm*ChiP and kanamycin B (hot pink) include hydrogen bond (white) and hydrophobic (blue) interaction. The key residues made hydrogen bond network of *Sm*ChiP and kanamycin B are Ser^{137} Arg^{18} Glu^{82} Arg^{318} Asp^{320} Asn^{416} Lys^{425} Arg^{299} and His^{135}, and hydrophobic interaction made by Trp^{16} and Tyr^{304}. The structures are visualized by PyMOL [20].

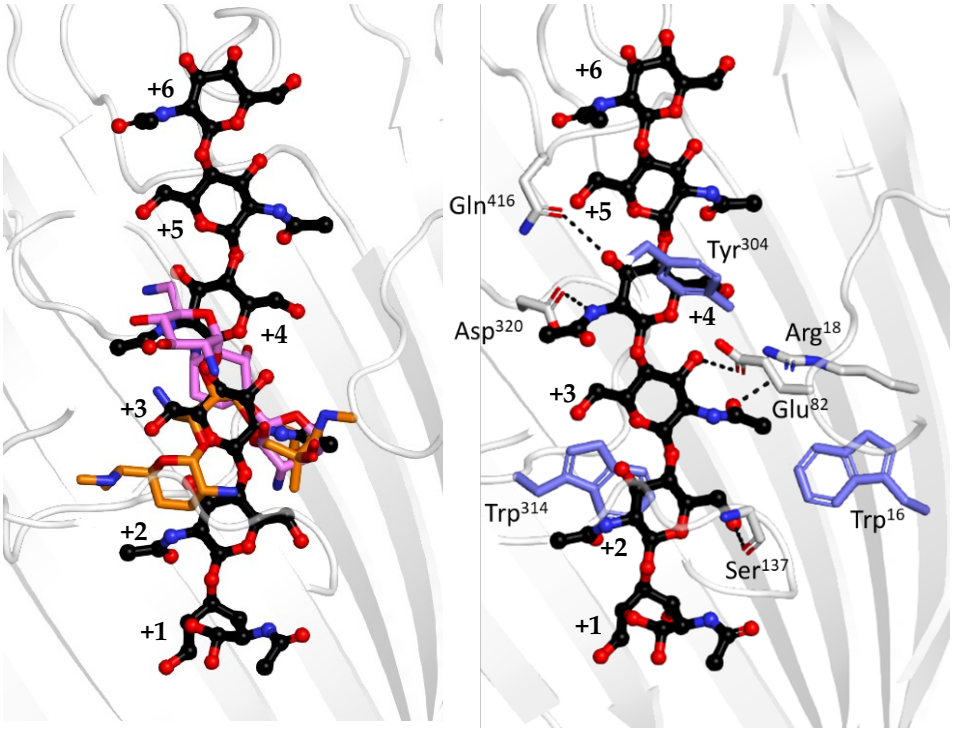


Figure 4. The comparison of aminoglycosides and chitohexaose from the unpublished crystal structure. (a) Two aminoglycosides were found at the area of GlcNAc affinity site of GlcNAc-2 to GlcNAc-4 with the ring C of antibiotics faced at the acetamido group of GlcNAc-3. (b) The interactions of *Sm*ChiP and $(GlcNAc)_6$ are from the unpublished data. GlcNAc-2 to GlcNAc-4 are interacted to the channel using hydrogen bond and hydrophobic interaction. The hydrogen bond are made by Ser^{137} (+2), Arg^{18} (+3), Glu^{82} (+3), Asp^{320} (+4), and Gln^{416} (+4), while hydrophobic interaction are stacked with the aromatic rings of Trp^{16} (+2), Trp^{314} (+2) and Tyr^{304} (+4).

4. Discussion

Chitoporin is an outer membrane porin which is specific to chitooligosaccharides. Previous studies showed that the chitinolytic machinery of *Sm* uses chitoporin (referred to as *Sm*ChiP in previous and this studies) for the chitooligosaccharide uptake. *Sm* was reported to possess several chitinolytic enzymes that help the bacterium to degrade and utilize chitin as its carbon source [21–25]. A previous study demonstrated the importance of *Sm*ChiP in the uptake of long chain of chitooligosaccharides such as chitotetraose ($(GlcNAc)_4$), chitopentaose ($(GlcNAc)_5$), and chitohexaose ($(GlcNAc)_6$) [26]. *Sm*ChiP had the amino acid sequences similar to that of *Ec*ChiP (about 70% identity) in the amino acid alignment [10,12,13]. Moreover, the function of *Ec*ChiP has been recently established [10,12]. *Ec*ChiP is the monomeric porin, which can stably inserted into the lipid bilayer with the average channel conductance of 0.55 nS in 1 M KCl electrolyte [10,12]. In this study, further application of chitoporin is presented. According to the antibiotics susceptibility studies, *Sm* type strain ATCC® 14756 was susceptible to only gentamicin when compared to the MIC values reported in the EUCAST table. Single channel studies of *Sm*ChiP in the presence of 1 mM aminoglycosides showed that only kanamycin B and gentamicin can interfere the current flow, as indicated by the transient drops of the currents when the antibiotic was added into the cis side of the chamber. The ion fluctuations were observed only at negative potential side because of the positive charge of the drug molecules. The structures of gentamicin and kanamycin B contain 5 positively-charged amine groups (**Figure 5**) [27–29]. In single channel experiments, the potential applied means the direction of electric field can assist the charged molecules to flow following the direction of the electric field. In this case, the negative potential applied to the trans side, therefore, the positive charged molecule would bring to the trans side according to their

polarity [8,30]. Hence, gentamicin and kanamycin B, which contained more positive group, would bring to trans side, and interact to the channel. Although, the transportation event needs further experiment, the titration of antibiotic concentration and varying potential applied, to be confirmed.

Furthermore, *Sm*ChiP interaction with gentamicin and kanamycin B were generated by docking simulation using unpublished data set of *Sm*ChiP from our previous work. The final best score showed the conformation of gentamicin and kanamycin B were similarly bond to the constriction area as same as the natural substrate, $(GlcNAc)_6$. The interaction analysis showed the same binding key residues including Ser^{137} Glu^{82} Arg^{318} Asp^{320} and Lys^{425} which are Ser^{137} Glu^{82} and Asp^{320} are the main residues that *Sm*ChiP made hydrogen bonds to the center of $(GlcNAc)_6$: Ser^{137} to GlcNAc-2, Glu^{82} to GlcNAc-3, and Asp^{320} to GlcNAc-4. These can conclude that *Sm*ChiP occupied inside the channel lumen at the constriction area. However, the crystal structures of *Sm*ChiP in complex with antibiotics will help to be confirm the interactions and the conformations of antibiotics inside the channel.

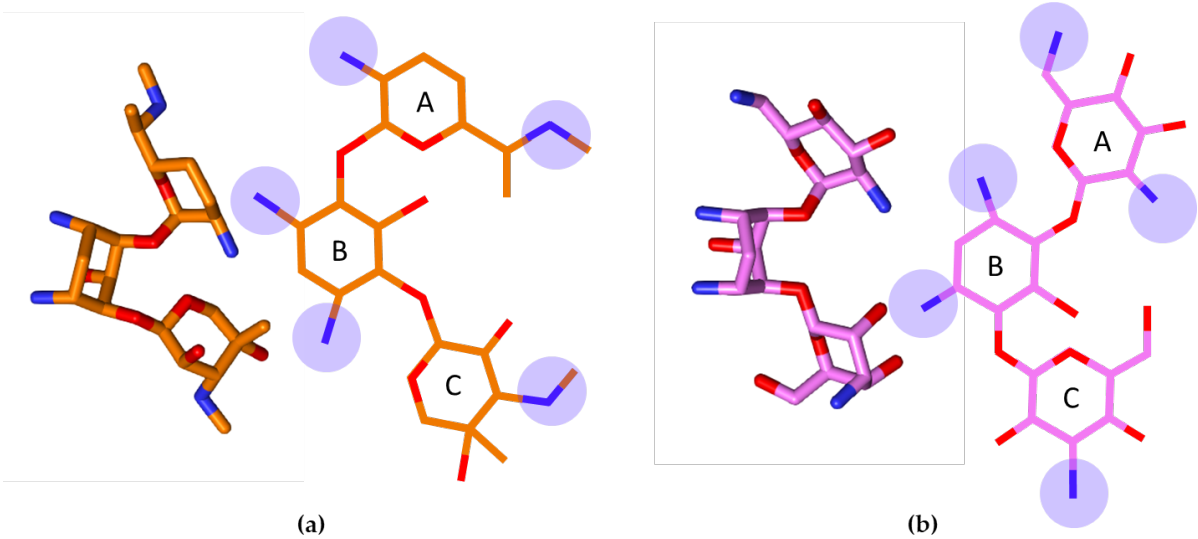


Figure 5. 3D-structure compared with 2D-structure of aminoglycosides. (a) Gentamicin and (b) Kanamycin B obtained from PubChem, gentamicin CID: 73295 and kanamycin B CID: 25245850. The structures contain three amino sugar ring, which ring B of these two antibiotics are 2-deoxystreptamine that linked glycosidic bond to other two amino sugar rings A and C.

5. Conclusions

The studies of antibiotic interactions to chitoporin from *S. marcescens*, *Sm*ChiP, indicated the two aminoglycosides can interact with the channel, including gentamicin and kanamycin B. Antibiotic susceptibility showed that *S. marcescens* was susceptible only towards gentamicin but not to other antibiotics. Although kanamycin B showed the ability to stop the cell growth, the bacterium did not exhibit significant susceptibility towards this antibiotic. These two antibiotics have similar structures, containing more amine groups, thus, interacted the channel at the applied negative potential. When molecular interactions were analyzed by Gold docking, the best conformation of gentamycin and kanamycin B were shown to fit in the constriction area of the channel and interacted with the channel with the same key residues as $(GlcNAc)_6$. Our agar disk assays showed that *Sm* was an opportunistic pathogen that is highly resistance against narrow spectrum antibiotics, including penicillin, cephalosporins, and fluoroquinolones. Our study that showed that *Sm* was susceptible to gentamycin, and perhaps kanamycin B. These two antibiotics were uptake through *Sm*ChiP, which could help to pave the way to develop further optimization to obtain much more effective antibiotics using gentamicin and kanamycin as the molecular scaffolds and understanding the interactions of *Sm*ChiP and antibiotics can provide a clear idea how to achieve a novel design of aminoglycoside antibiotics that can be effective against *Sm*.

Supplementary Materials: All materials contained in the article.

Author Contributions: R.A. conducted all experiments, visualized, and analyzed the data, wrote the article draft and prepared all figures. W.S. reviewed and edited the manuscript, guided, and supported whole project completion.

All authors have read and agreed to the published version of the manuscript.

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