Single-channel characterization of the chitooligosaccharide transporter chitoporin (SmChiP) from the opportunistic pathogen *Serratia marcescens*

Received for publication, May 1, 2022, and in revised form, September 8, 2022. Published, Papers in Press, September 14, 2022, J. Biol. Chem. (2022) 298(10) 102487

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Edited by Robert Haltiwanger

*Serratia marcescens* is an opportunistic pathogen that can utilize chitin as a carbon source, through its ability to produce chitin-degrading enzymes to digest chitin and membrane transporters to transport the degradation products (chitooligosaccharides) into the cells. Further characterization of these proteins is important to understand details of chitin metabolism. Here, we investigate the properties and function of the *S. marcescens* chitoporin, namely SmChiP, a chitooligosaccharide transporter. We show that SmChiP is a monomeric porin that forms a stable channel in artificial phospholipid membranes, with an average single-channel conductance of 0.5 ± 0.02 nS in 1 M KCl electrolyte. Additionally, we demonstrated that SmChiP allowed the passage of small molecules with a size exclusion limit of <300 Da and exhibited substrate specificity toward chitooligosaccharides, both in membrane and detergent-solubilized forms. We found that SmChiP interacted strongly with chitopentaose ($K_d = 23 ± 2.0 \mu M$) and chitohexaose ($K_d = 17 ± 0.6 \mu M$) but did not recognize nonchitose oligosaccharides (maltotriose and cellotriose). Given that *S. marcescens* can use chitin as a primary energy source, SmChiP may serve as a target for further development of nutrient-based antimicrobial therapies directed against multidrug antibiotic-resistant *S. marcescens* infections.

*Serratia marcescens* is a facultative Gram-negative, soil-borne bacterium (1) that frequently causes outbreaks of community- or hospital-acquired infections in adults and children (2, 3). *S. marcescens* may cause respiratory infections (4, 5), meningitis (6, 7), and urinary tract infections (8). The microorganism is highly resistant to most antimicrobial agents (9–11). Numerous strains of *S. marcescens* possess quorum-sensing regulated virulence factors, which are endogenous plasmids that carry antibiotic-resistance genes (12). Most strains also carry highly effective ABC-type efflux pumps (13). In addition, the cell envelope of *S. marcescens* has a complex lipopolysaccharide layer, which increases the bacterial membrane barrier. *S. marcescens* also forms biofilms (14–17). Treatment of *S. marcescens* infections can be difficult, owing to its intrinsic resistance to most antibiotics, including amoxicillin, ampicillin, the first generation of cephalosporins and carbapenems, and some fluoroquinolones (10, 18, 19). In severe cases, patients require intensive medical treatments using fourth-generation cephalosporins or piperacillin/tazobactam (20).

*S. marcescens* can utilize various types of organic material as its carbon source and can produce chitin-active enzymes to hydrolyze chitin materials and use them as an energy source (21). Chitin breakdown is initiated by a chitin-active lytic polysaccharide monooxygenase that breaks the chitin polysaccharide chain into chitin fragments by oxidative cleavage (22, 23). The oxidized chitin fragments are hydrolyzed further by chitinases (21, 22, 24–28) to short-chain chitooligosaccharides, which are transported into the periplasm of the bacterial cell through outer membrane (OM) pores. Previous studies reported the existence of several porins in the OM of *S. marcescens*, including Omp1, Omp2, Omp3, OmpF, and OmpC. These general diffusion pores take up small, hydrophilic molecules by passive diffusion (29, 30). On the other hand, a disaccharide (chitobiose) and higher molecular weight chitooligosaccharides, which cannot pass through the general diffusion pore, require a chitooligosaccharide-specific porin (ChiP) for their entry into the periplasm (24). Watanabe et al. (25) previously identified the *chiP* gene encoding ChiP (later named SmChiP) as part of the *chiPQ-ctb* gene cluster in the genome of *S. marcescens* 2170. The expression of *chiP* mRNA is controlled by the nontranslated *chiX* small RNA that binds to the gene’s 5′-untranslated region, which contains a 17-nucleotide Shine-Dalgarno sequence preceding the *chiPQ-ctb* gene cluster. The same study also demonstrated that the ΔChiP mutant had a drastically reduced growth comparing to the growth of the wildtype, confirming that ChiP played an important role in cell survival. We previously identified and characterized a chitooligosaccharide-specific porin, namely...
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SmChiP, from *S. marcescens* (31). In this report, we employed time-resolved single channel electrophysiology to examine the ion-conducting properties and substrate specificity of SmChiP. We also carried out *in vivo* cell studies to demonstrate that *S. marcescens* can employ chitooligosaccharides as its primary carbon source.

**Results**

**Sequence analysis and AlphaFold2 structural prediction**

We previously reported that some characterized chitoporins (ChiPs) exist as trimers and others as monomers, depending on the organism. For example, *VfChiP* identified from *Vibrio furnissii* (32), *VhChiP* from *Vibrio campbellii* (formerly classified as *Vibrio harveyi*) type strain ATCC BAA 1116 (33), and *VcChiP* from *Vibrio cholera* type strain O1 (34) are each composed of three identical subunits, while *EcChiP* from *Escherichia coli* is a monomeric porin (35, 36).

**Figure 1** shows structure-based sequence alignment of monomeric ChiPs, including SmChiP and EcChiP, in comparison with trimeric ChiPs, including *VhChiP* and *VcChiP*. From the sequence alignment, SmChiP has high (77%) sequence identity with EcChiP, but low (17%) sequence identity with the trimeric ChiPs. The secondary structure elements of SmChiP are similar to those of EcChiP but different from those of the trimeric ChiPs. Overall, monomeric ChiPs have a single-barrel structure composed of 18 β-strands connected by eight extracellular loops, while *VhChiP* and other trimeric ChiPs contain 16 β-strands connected by eight extracellular loops. Loop L3 of trimeric ChiPs, which is typically identified as the pore-confining loop, is exceptionally long when compared with that of the monomeric ChiPs, while loops L2 and L6 of monomeric ChiPs are longer than those in the trimers. The N-terminal segment that is characteristic of trimeric ChiPs is longer than that of the monomeric ChiPs. Notably, the N-terminal segment of *VhChiP* contains a short helix that serves as the plug that regulates the open and closed states of the *VhChiP* pores (37). This N-plug is unique to *VhChiP* and is not present in other ChiPs (Fig. 1).

Figure 2 shows the AlphaFold2-predicted structure of SmChiP, compared with the crystal structure of the native *VhChiP* (PDB id: 5MDQ) (38). Figure 2A shows the side view (left panel) and top view (right panel) of *VhChiP*, consisting of three identical trimers, each of which contains the characteristic N-terminal plug. 19 amino acids long, with a short helix. In the closed channel, the N-plug of each monomer was found to plug the bottom half of the neighboring pore, causing the channel to close. The most prominent loop L3 (labeled green) contains three short helices, which folded inside the central part of the protein pore. This loop was the most important loop in *VhChiP*, in that it contained several amino acid residues that bound to the sugar substrate (chitohexaose) (38). Figure 2B (left panel) shows the side view of the monomeric SmChiP barrel, lacking the N-plug. Although loop L3 of SmChiP also protruded into the protein pore, it was not helical, leaving more space inside the channel interior (Fig. 2B, right panel). Superimposition of SmChiP onto one monomer of *VhChiP* yielded an R.M.S.D. of 3.75 Å for 940 atoms, reflecting large differences in the amino acid arrangement inside the two.
channels. We found a few residues that are conserved but were unable to identify the amino acid residues inside the SmChiP pore that could form the substrate binding sites, due to the high diversity in amino acid sequences between SmChiP and VhChiP.

Determination of the molecular weight of native SmChiP

The native state of the OM-expressed SmChiP was determined by size exclusion chromatography. Figure 3 shows the elution profiles of the protein standards together with SmChiP, using a HiPrep 26/60 prepacked Sephacryl S-300 column. SmChiP was eluted at a position between ovalbumin (43 kDa) and bovine serum albumin (66 kDa) (Fig. 3A). The eluted fractions obtained from the A$_{280}$ peak were pooled and analyzed by SDS-PAGE. Figure 3A (inset) shows a Coomassie-stained protein band, which migrated at about 45 kDa, consistent with the apparent molecular mass of 50 kDa of SmChiP determined from the distribution coefficient ($K_{av}$) (Fig. 3B) and confirming that SmChiP is a monomer.

Single-channel electrophysiology

Single-channel measurements were carried out with lipid bilayer measurements (BLM) SmChiP reconstituted in 1,2-diphytanoyl-sn-glycero-3-phosphocholine membranes, and ion flow through the membrane-embedded SmChiP was recorded at different applied potentials. Figure 4, A and B show representative ion current traces acquired over 2000 ms at +100 mV and −100 mV, respectively. The channel was also found to be constantly open within a wide range of applied transmembrane potentials from ±25 mV to ±150 mV (data not shown).

Ion traces acquired from a single SmChiP channel exhibited single-step openings with no subconductance or gating, at both negative and positive potentials. A current magnitude of approximately 50 pA was consistently observed under the applied potentials of ±100 mV in 1 M KCl electrolyte, giving the average single channel conductance of 0.5 nS ($G = 0.5$ nS). This conductance value of SmChiP is about one-third of that of trimeric VhChiP ($G = 1.8 ± 0.3$ nS) (39), consistent with the monomeric structure of the SmChiP channel. Multichannel reconstitution experiments (Fig. 4C) further confirmed the mean channel conductance of 0.54 ± 0.2 nS from 60 independent channel insertions, obtained from Gaussian distribution fitting of the histogram (Fig. 4D). The channel conductance obtained from the I-V plot (Fig. 4E, for nine independent insertions) was 0.54 ± 0.01 nS, which was essentially identical with the value obtained from the multichannel insertions shown in Figure 4C.

Sugar selectivity of SmChiP at single-channel level

In this series of experiments, we examined the substrate specificity of SmChiP by exposing the channel to different chitooligosaccharides and examining their transient blocking behavior. In the absence of sugar, SmChiP was fully open, allowing a steady ionic current over the entire recording time of 2 min at both applied negative and positive potentials. Figure 5A shows a representative ion trace of an empty channel, which had ionic conductance of −50 pA at −100 mV.
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Figure 3. Molecular weight determination by size-exclusion chromatography. A, standard proteins and SmChiP were resolved on a HiPrep 16/60 Sephacryl S-200 HR prepacked column under the conditions described in the text. B, the molecular weight of SmChiP was estimated from the plot of the distribution coefficient ($K_{av}$) versus log$_{10}$ MW of four well-resolved standard proteins: ribonuclease, carbonic anhydrase, ovalbumin, and BSA. Control peaks were DNP-lysine, which was applied to determine the total volume of the GF column. The inset shows migration of SmChiP on SDS-PAGE, in comparison with that of the standard proteins. The protein bands were stained with Coomassie blue G-250 and photographed in monochrome. GF, gel filtration; SmChiP, Serratia marcescens chitoporin.

Figure 4. Pore-forming properties of SmChiP in artificial lipid membranes. Lipid bilayer measurements (BLM) were formed by a lowering-and-raising technique, using 5 mg ml$^{-1}$ DPhPC bathed on either side with 1 M KCl in 20 mM Hepes, pH 7.4. The protein was always added to the cis side of the chamber. A, current trace at +100 mV, (B) current trace at −100 mV, and (C) multiple channel insertion. D, histogram analysis of channel conductance observed from 60 independent channel insertions. The distribution of the conductance profile was fitted to a Gaussian curve by Clampfit v.10.4. DPhPC, 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine; SmChiP, Serratia marcescens chitoporin.
The addition of 80 μM D-GlcNAc (Fig. 5B) on either side of the chamber did not disturb the ion flow, while the addition of chitobiose at the same concentration (Fig. 5C) caused slight flickering of the current trace. Disturbance of the ion flow was seen when the channel was exposed to chitotriose. Figure 5D shows that the sugar molecules occluded the channel, causing short-lived blocking events throughout the recording time. The addition of chitotetraose (Fig. 5E) also yielded frequent, full blocking events. A similar blocking pattern was observed with chitopentaose, with more blocking events than were detected with chitotriose and chitotetraose (Fig. 5F). In the case of chitohexaose, although a lower number of blocking events was seen, they were full and long-lived (Fig. 5G).

The average residence time \( (\tau_c) \) for channel blocking by chitotriose cannot be evaluated with confidence because of the limit of time resolution of our BLM instrument (<100 μS). However, the residence times for chitotetraose, chitopentaose, and chitohexaose were estimated to be 0.3, 1.3, and 6.0 ms, respectively.

Histogram analysis confirmed the blocking characteristics of individual sugar species (Fig. 5, A–I, insets). D-GlcNAc, chitobiose, and chitotriose (Fig. 5, B–D, insets) did not alter the amplitude of the ionic current, which corresponded to the fully ‘open’ state of the channel (50 pA at +100 mV). On the other hand, transient blockings caused a discrete reduction of the ion current to zero when 80 μM of chitotetraose, chitopentaose, or chitohexaose was added. The fraction of the porin at zero current (labeled ‘closed’) increased as the chain length increased, indicating that long-chain chitooligosaccharides induced more complete blocking of the protein subunits (Fig. 5, E–G, insets).

**Bulk permeation of small monosaccharides and chitooligosaccharides through SmChiP**

Liposome swelling assays were carried out to examine the permeation of bulk sugars through the SmChiP-reconstituted proteoliposomes. Figure 6A shows the swelling rate with small sugar molecules of molecular weights 180 to 600 Da. The permeation rate of the tested sugar was estimated relative to that of the smallest sugar (D-arabinose, MW = 150), which was set to 100%. The isotonic concentration was determined to be...
70 mM, using the impermeant sugar raffinose. All monosaccharides, including D-glucose, D-galactose, and D-mannose (all of MW = 180 kDa) and D-GlcNAc (MW = 222 Da), permeated the liposomes at relative rates of nearly 100%, while the disaccharides D-sucrose (MW = 342), D-maltose (MW = 360), and D-melezitose (MW = 522) were completely impermeant, reflecting a pore constriction limit of <300 Da. The permeation rates of different chitooligosaccharides were also tested. Figure 6B suggests that all chitooligosaccharides could permeate through the liposomes, even at a concentration as low as 2.5 mM. Chitotetraose, chitopentaose, and chitohexaose had slightly higher permeation rates than chitobiose and chitotriose. However, maltohexaose did not permeate through SmChiP at all.

**Substrate binding affinity of SmChiP**

Isothermal microcalorimetric (ITC) titrations were carried out with SmChiP in solution with two preferred substrates (chitohexaose and chitopentaose) and were compared to the data with a nonchitin oligosaccharide (maltohexaose).

Figure 7A shows the ITC thermograms obtained from titrating chitohexaose to SmChiP, and Figure 7B shows the theoretical fit of the normalized, integrated heat using a one-site binding model available in MicroCal PEAQ-ITC Analysis Software. Figure 7, C and D show the isothermal binding thermogram and the corresponding curve fit for chitopentaose. The equilibrium dissociation constant ($K_d$) was estimated to be $17 \pm 0.6 \mu M$ for chitohexaose and $23 \pm 2.0 \mu M$ for chitopentaose. Figure 7E is the isothermal titration profile of maltohexaose, showing no heat release (<0.1 μcal per injection) and yielding no integrated heat change (Fig. 7F), as a result of no binding.

**Growth of S. marcescens on different carbon sources**

*S. marcescens* was grown on M9 minimal medium (MM) supplemented with three different carbon sources: D-glucose, a chitooligosaccharide mixture, and chitosan oligomers, and the growth was monitored at different time points from 0 to 96 h. Figure 8 shows the rapid growth of *S. marcescens* during the log phase of incubation (within 24 h), when D-glucose and chitin oligomers were used as the carbon source. The growth rate after entering the stationary phase declined less slowly with the cells grown on chitooligosaccharides than on D-glucose. *S. marcescens* could barely grow on MM supplemented with chitosan oligomers and was unable to grow on MM with no supplementary carbon source.

**Discussion**

The *chiP* gene encoding SmChiP from *S. marcescens* 2170 was first identified as part of the *yfhMN-ctp* gene cluster. Later, this was referred to as the *chiPQ-ctb* cluster (40). The same study also generated the *S. marcescens* mutants lacking the *chiP*, *chiX*, and *chiQ* genes. Deletions of these target genes affected the growth of the bacterium. In particular, the △*chiP* mutant showed significantly lower ability to grow on medium supplemented with colloidal chitin and (GlcNAc)$_2$, and no growth on medium containing (GlcNAc)$_3$. Subsequent studies demonstrated that the *chiX* small RNA controlled the expression of chitin-degrading enzymes (chitinases, chitobiase, and N-acetylglucosaminidase), chitin-binding protein (CBP21)
and a chitooligosaccharide-transporting porin (later named SmChiP) (33, 40). Nonetheless, no detailed functional and structural characterization of SmChiP has been reported to date. In the present study, we heterologously expressed SmChiP in the E. coli system. The pore-forming properties of SmChiP in lipid membranes were elucidated at the single-molecule level. In contrast to the well-characterized trimeric VhChiP from V. campbellii (formerly V. harveyi) (33, 38, 40) and VcChiP from Vibrio cholerae (34), SmChiP was shown to be a member of the class of monomeric porins, like the closely related EcChiP (35). Although most porins form trimers, a few were shown to be monomeric, including Pseudomonas aeruginosa OCCDs and OCCks (formerly referred as OprD porins) (42, 43) and E. coli OmpG (44).

Figure 7. Binding studies of SmChiP with three oligosaccharides. Microcalorimetric titrations of SmChiP with oligosaccharides, showing ITC profiles corresponding to the binding of (A) chitohexaose, (C) chitopentaose, and (E) maltohexaose to SmChiP. B, integrated curve fitting for heat of binding of chitohexaose, (D) chitopentaose and (F) maltohexaose. ITC, isothermal microcalorimetric; SmChiP, Serratia marcescens chitoporin.

Bulk permeability of different sugars through SmChiP, as determined by a liposome swelling assay, provided an estimated size exclusion limit of about 300 Da. In our study, only monosaccharides were able to pass through the protein pore by general diffusion. SmChiP exhibited clear substrate specificity, being completely impermeable to nonchitin oligosaccharides. However, BLM results showed that chitobiose and longer-chain chitooligosaccharides (chitotriose, -tetraose, -pentaose and -hexaose) could permeate through SmChiP, despite their MWs exceeding its size exclusion limit. We recently showed that the C2-acetamido functionality on the GlcNAc units of the chitooligosaccharide chain served as a molecular footprint for sugar-channel recognition (45). SmChiP was shown to have no binding affinity for chitosan oligomers (deacetylated chitooligosaccharides), since these sugars lack the C2-acetamido functional groups. Our thermodynamic data obtained from ITC experiments confirmed that the binding affinity for the most highly preferred substrate, chitohexaose ($K_d = 17 \mu M$ or $K = 60,000 \text{ M}^{-1}$) was 4.2-fold and 8.4-fold lower than for chitohexaose binding to EcChiP ($K = 250,000 \text{ M}^{-1}$) (35) and to VhChiP ($K = 500,000 \text{ M}^{-1}$), respectively. The ITC data confirmed that SmChiP had weaker substrate-binding affinity than EcChiP (35) and VhChiP (40).
Single-channel recordings suggested that SmChiP could form a highly stable channel over a wide range of applied external potentials (±25 to ±150 mV), with neither gating nor stepwise closure. The average single-channel conductance of SmChiP ($G = 0.5 \pm 0.2$ nS) was approximately one-third of the mean conductance reported for trimeric VhChiP ($G = 1.8 \pm 0.3$ nS) (40), which confirmed the monomeric state of SmChiP.

Cell growth assays showed that S. marcescens could grow on chitin oligosaccharides, owing to its ability to digest chitinous materials and transport the degradation products through SmChiP into the cell.

Figure 9 is a schematic illustration of chitin processing by the OM and inner membrane (IM) of S. marcescens, suggested by Watanabe et al. (22, 23). Chitin is initially digested by chitinases, generating chitooligosaccharides of various lengths as the initial products, and finally further generating (GlcNAc)$_2$ as the major product (dark arrow), along with the monosaccharide (D-GlcNAc) as a minor product (dashed arrow). The chitin degradation products enter the periplasm by two possible routes: (GlcNAc)$_2$ and chitooligosaccharides through SmChiP and GlcNAc through a general diffusion porin. (GlcNAc)$_2$ in the periplasm was suggested to be transported further through the IM by a specific (GlcNAc)$_2$-specific enzyme IIC, encoded by the chb operon (22). On the other hand, GlcNAc is further transported through the IM by a GlcNAc-specific phosphoenolpyruvate:carbohydrate phosphotransferase system transporter. (GlcNAc)$_2$ and GlcNAc entering the cytoplasm via phosphoenolpyruvate:carbohydrate phosphotransferase system are phosphorylated during transport to (GlcNAc)$_2$-P and GlcNAc-6P, which are further metabolized in the cytoplasm (46).

In conclusion, this study elucidates the physiological function of a chitooligosaccharide-specific porin from the Serratia system and provides an understanding of how the Serratia bacteria can utilize chitin as their carbon source. Given that SmChiP is the molecular gateway for nutrient uptake, it may serve as an excellent protein target for the strategic design of effective antimicrobial agents with a novel mode of action, such as the chemoenzymatic synthesis of chitooligosaccharide-based compounds that can inhibit Serratia infections.

**Experimental procedures**

**Bacterial strains and vectors**

The pET23a(+) expression vector, carrying the S. marcescens ChiP gene, was obtained from GenScript USA Inc. E. coli strain DH5α, used for plasmid preparations, was obtained from Invitrogen (Gibthai Company, Ltd). E. coli BL21 (DE3) Omp8 Rosetta strain, lacking the major endogenous Omps (OmpF, OmpC, OmpA, and LamB) was a gift from Professor Dr Roland Benz, Jacobs University Bremen, Germany. Chitooligosaccharides were purchased from Dextra Laboratories and Megazyme.

**Structure-based sequence alignment and AlphaFold2 structural prediction**

Structure-based sequence alignment was performed for representative ChiPs from S. marcescens (SmChiP; protein id: A0A0PBS3), E. coli (EcChiP, protein id: P75733), V. cholerae (VcChiP, protein id: Q9KTD0), and V. harveyi, now classified as V. campbellii (VhChiP, protein id: L0RYU0). The corresponding sequences were retrieved from the UniProtKB database.

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Figure 8. Growth of S. marcescens on various carbon sources. S. marcescens were grown on M9 minimal medium supplemented with 0.5% (w/v) chitooligosaccharide mixture, D-glucose, or chitosan oligomers. The growth rate was monitored at different time points of incubation up to 96 h, at 26 °C.

Figure 9. Chitin utilization by S. marcescens. GlcNAc and (GlcNAc)$_2$-6 are chitooligosaccharides generated by the cleavage of chitin by chitinase. GlcNCase is a periplasmic β-N-acetylglucosaminidase and GlcN is glucosamine. Gray arrows indicate the main enzymic route, while dashed arrows indicate minor enzymic routes. Red arrows indicate translocation steps. IM, inner membrane; OM, outer membrane; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system.
database (https://www.uniprot.org/uniprot/), aligned by Muscle alignment (47) and displayed in Jalview v. 2.11.2.1. The 2D structural elements of monomeric SmChiP were constructed from AlphaFold2 (https://alphafold.ebi.ac.uk/), while the 2D structural elements of trimeric VhChiP were constructed from the crystal structure of the native form (PDB id: 5MDO).

**Recombinant expression, cell wall extraction, and protein purification**

Cloning of the chiP gene encoding SmChiP and purification of the recombinant protein were described in our previous report (31). Briefly, the overnight culture of transformed cells was transferred to Luria-Bertani (LB) broth containing 100 μg ml⁻¹ ampicillin and 25 μg ml⁻¹ kanamycin and grown at 25 + 1 °C until A₆₀₀ ~0.6 to 0.8. SmChiP expression was then induced with 0.5 mM of isopropyl thio-β-D-galactoside for 6 h at 37 °C. For protein purification, the cell pellet, collected after centrifugation and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 2.5 mM MgCl₂, 0.1 mM CaCl₂), containing 10 μg ml⁻¹ RNase A and 10 μg ml⁻¹ DNase I, was subjected to high-speed ultrasonic homogenization (EmulsiFlex-C3). Cell walls were extracted by incubating the crude extract in 2% (w/v) SDS solution at 50 °C for 60 min. After centrifugation at 100,000g at 4 °C for 1 h, the cell wall fraction (pellet) was extracted twice with 2.5% (v/v) octyl-POE (n-octylpolyoxyethylene; ALEXIS Biochemicals). After centrifugation at the same speed, the supernatant containing OM-expressed SmChiP was dialyzed thoroughly against 20 mM potassium phosphate buffer, pH 7.4, containing 0.05% (v/v) lauryldimethylamine oxide (Sigma-Aldrich Pte Ltd), and purified by ion-exchange chromatography on a Hitrap Q HP prepacked column (1 cm Ø × 5 cm L), followed by gel filtration chromatography on a HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare Life Sciences). The purity of the SmChiP fractions obtained after the gel filtration step was verified by SDS-PAGE, and the protein concentration of the purified SmChiP was estimated using the Novagen BCA protein assay kit (EMD Chemicals Inc).

**Molecular weight determination of native SmChiP**

The molecular weight of SmChiP in its native state was determined by size-exclusion chromatography. Standard proteins of known molecular weight were resolved on a HiPrep 16/60 Sephacryl S-200 HR prepacked column (GE Healthcare Life Sciences) under the conditions described above. Dextran-2000 was used to obtain the void volume (V₀), while DNP-lysine was used to calculate the volume of the stationary phase (Vₑ). With the elution volume of each protein sample denoted Vₑ, the elution of the protein sample is described by the distribution coefficient (Kₑ) defined in Equation 1 (48):

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Kₑ = \frac{Vₑ - V₀}{Vₑ - V₁}
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A calibration curve was created by plotting Kₑ versus logarithmic values of the corresponding molecular weights of the standard proteins and was used to estimate the molecular weight of SmChiP. The standard proteins used in this experiment were ferritin (440 kDa), catalase (250 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa).

**Single-channel electrophysiology**

Montal-Mueller type solvent-free bilayer (49) formation was performed using 5 mg ml⁻¹ 1,2-dihytyanoyl-sn-glycero-3-phosphatidylcholine (Avanti Polar Lipids) in n-pentane. First, a 25-mm-thick Teflon film with an aperture of 50 to 100 mm was sandwiched between the two chambers of a cuvette, and the aperture was prepped with a few microliters of 1% (v/v) hexadecane in hexane. The chambers were filled with 1 M KCl in 20 mM Heps, pH 7.4, and BLM experiments were carried out at 25 °C. A planar bilayer was formed across the aperture by lowering and raising the liquid level (33). Ionic currents were detected using Ag/AgCl electrodes, with the reference electrode connected to the cis side of the membrane (ground) and the working electrode connected to the head-stage of an Axopatch 200B amplifier (Axon Instruments). Single-channel measurements were performed in the voltage clamp mode and digitized using the Axon Digidata 1550 digitizer, and the data acquisition was performed using Clampex software (Axon Instruments). Using a low-pass Bessel filter with a sampling frequency of 50 kHz, the traces obtained were filtered at 10 kHz. Single channel analyses were performed using Clampfit software (all from Molecular Devices). Single protein channels were reconstituted in lipid, and fully open channels of SmChiP were titrated with discrete concentrations of chitooligosaccharides at the cis or trans side of the chamber. Fluctuations of ion flow produced by sugar diffusion through the inserted channel were usually recorded for 2 min at different transmembrane potentials of ±25 to ±100 mV. Multiple channel insertion experiments were operated with a patch-clamp amplifier connected to a two-electrode bilayer head-stage (PC-ONE plus PC-ONE-50; Dagan Corp) together with an A/D converter (LIH 1600, HEKA Elektronik) that was operated using PULSE program (HEKA Elektronik).

**Liposome swelling experiments**

The SmChiP-reconstituted proteoliposomes were prepared as described elsewhere (35, 50). Soybean 1-α-phosphatidylcholine [20 mg ml⁻¹, (Sigma) freshly prepared in chloroform] was used to form multilamellar liposomes. For the preparation of proteoliposomes, 200 μg of SmChiP was reconstituted into 200 μl of the liposome suspension by sonication, and then 17% (w/v) dextran (40 kDa) was entrapped in the proteoliposomes. D-Raffinose solutions were prepared in 20 mM potassium phosphate buffer, pH 7.4, to obtain concentrations of 40, 50, 60, and 70 mM for determination of the isotonic solute concentration. This value was then used for the adjustment of the isotonic concentration for other solutes. To carry out a liposome-swelling assay, 25 μl of the proteoliposome suspension was added to 600 μl of sugar solution, and changes in

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Chitoporin from Serratia marcescens

absorbance at 500 nm were monitored immediately. The apparent absorbance change over the first 60 s was used to estimate the swelling rate (s⁻¹) following the equation \( \Phi = (1/A_i)\Delta A/dt \) in which \( \Phi \) is the swelling rate, \( A_i \) is the initial absorbance, and \( \Delta A/dt \) is the rate of absorbance change during the first 60 s. The swelling rate for each sugar was normalized by setting the rate of L-arabinose (150 Da) to 100%. The values presented are averages from three independent determinations. Protein-free liposomes and proteoliposomes without sugars were used as negative controls. The sugars tested were D-glucose (180 Da), D-mannose (180 Da), D-galactose (180 Da), N-acetylglucosamine (GlcNAc) (221 Da), D-sucrose (342 Da), D-melezitose (522 Da), GlcNAc₂ (424 Da), GlcNAc₃ (628 Da), GlcNAc₄ (830 Da), GlcNAc₅ (1034 Da), GlcNAc₆ (1237 Da), and maltodextrins.

Chitooligosaccharide-induced growth of S. marcescens

*S. marcescens* was streaked onto LB agar plates, then incubated at 26 °C overnight. A single colony was picked and inoculated into LB medium for gentle shaking at 26 °C overnight. The bacterial cells were grown in M9 minimal medium (26 mM Na₂HPO₄, 22 mM KH₂PO₄, 19 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and 8.6 mM NaCl), supplemented with various carbon sources (D-glucose, chitosan oligomers or chitooligosaccharides), all at 0.5% (w/v). Cells (150 μl) were collected to measure \( A_{600} \) in a microtiter plate during each time course (0–96 h). Cell growth was monitored with a microplate reader model MULTISKAN Sky (Thermo Fisher Scientific). The number of cells (cfu.ml⁻¹) was calculated from \( A_{600} (0.1 \text{ A}_{600} = \times 10^8 \text{ cfu.ml}^{-1}) \) (51).

Data availability

The BLM and ITC datasets for this study can be found in the following URL link: https://drive.google.com/drive/folders/1qh5qDiOhLt0-c18cgT41XgZ42AY8jyA4.

Acknowledgments—We would like to acknowledge the help of Dr David Apps, University of Edinburgh, Scotland for critical readings of the manuscript. We would also like to thank Professor Mathias Winterhalter, Jacobs University Bremen, Germany, for providing the BLM setup for single channel measurements.

Author contributions—H. S. M. S., S. K., and R. A. methodology; H. S. M. S., S. K., and R. A. data curation; H. S. M. S., S. K., and R. A. writing-original draft; H. S. M. S., S. K., and R. A. visualization; H. S. M. S., S. K., and R. A. investigation; H. S. M. S. formal analysis; T. W. and W. S. conceptualization; T. W. and W. S. writing-review and editing; W. S. supervision; W. S. validation.

Funding and additional information—This research was funded by Thailand Science Research and Innovation (TSRI) and Vidyasirimedhi Institute of Science and Technology (VISTEC) (BRG6610008). H. S. M. S. received PhD funding from Suranaree University of Technology (SUT) through an OGRG grant.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ChiPs, chitoporins; GlcNAc, N-acetylglucosamine; (GlcNAc)₂, chitobiose; (GlcNAc)₃, n >2, chitooligosaccharides; IM, inner membrane; ITC, isothermal microcalorimetric; \( K_d \), equilibrium dissociation constant (μM); MM, M9 minimal medium; OM, outer membrane; SmChuP, *Serratia marcescens* chitoporin.

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