Single-channel properties, sugar specificity, and role of chitoporin in adaptive survival of *Vibrio cholerae* type strain O1

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*Vibrio cholerae* is a Gram-negative, facultative anaerobic bacterial species that causes serious disease and can grow on various carbon sources, including chitin polysaccharides. In saltwater, its attachment to chitin surfaces not only serves as the initial step of nutrient recruitment but is also a crucial mechanism underlying cholera epidemics. In this study, we report the first characterization of a chitooligosaccharide-specific chitoporin, VcChiP, from the cell envelope of the *V. cholerae* type strain O1. We modeled the structure of VcChiP, revealing a trimeric cylinder that forms single channels in phospholipid bilayers. The membrane-reconstituted VcChiP channel was highly dynamic and voltage induced. Substate openings O1′, O2′, and O3′, between the fully open states O1, O2, and O3, were polarity selective, with nonohmic conductance profiles. Results of liposome-swelling assays suggested that VcChiP can transport monosaccharides, as well as chitooligosaccharides, but not other oligosaccharides. Of note, an outer-membrane porin (omp)-deficient strain of *Escherichia coli* expressing heterologous VcChiP could grow on M9 minimal medium supplemented with small chitooligosaccharides. These results support a crucial role of chitoporin in the adaptive survival of bacteria on chitinous nutrients. Our findings also suggest a promising means of vaccine development based on surface-exposed outer-membrane proteins and the design of novel anticholera agents based on chitooligosaccharide-mimicking analogs.

*Vibrio cholerae*, a Gram-negative bacterium of the genus *Vibrio*, is a human pathogen causing the life-threatening waterborne disease cholera. Endemics and epidemics of cholera have been reported to be a serious global health issue, especially in countries with poor sanitary provisions (1–4). The World Health Organization has estimated 1.3 to 4.0 million cases of cholera annually, with 21,000 to 143,000 deaths worldwide (https://www.who.int/news-room/fact-sheets/detail/cholera, Accessed, January 17, 2019). The main symptoms of *V. cholerae* infection are watery diarrhea and vomiting, causing dehydration, and the condition is life-threatening if not treated promptly. *V. cholerae* is a facultative anaerobe that employs both respiratory and fermentative metabolism using different carbon sources. In aquatic environments, they can grow on the chitin surfaces of abundant zooplankton, using them as their sole carbon source (5). Chitin-*V. cholerae* interactions are anticipated to be involved at multiple levels on a hierarchical scale and to play vital roles in the survival and pathogenicity of the bacteria (1, 6, 7). The association of *V. cholerae* with chitin has been shown to increase the level of resistance of the microbe to gastric acid during transit through the stomach (8, 9). It has been reported that the gene expression profiles of *V. cholerae* are different during the human and environmental stages of their life cycle, allowing them to live under fluctuating conditions, such as temperature shifts, osmotic stress, and nutrient limitation, in the aquatic environment, as well as to reside inside human hosts (4). *V. cholerae* usually forms biofilms in both planktonic and colonized states, and, as a consequence, increasing the density of hyperinfective cells as well as elevating the levels of transmission and dissemination (2, 10).

On the attachment of *V. cholerae* to chitin, the polysaccharide is broken down by extracellular chitinases into small chitooligomers, which are then transported through the cell wall of the bacteria through one of the protein channels (porins) for further metabolism as the source of energy (11, 12). Several outer-membrane porins from *V. cholerae* have been identified and characterized (13). Of these, OmpU (38 kDa) and OmpT (40 kDa) were shown to be voltage-sensitive and cation-selective (14). Both OmpT and OmpU were later shown to allow the passage of charged amino acids and antibiotic molecules (15). Another *V. cholerae* porin, identified as VCA1008, was shown to be selective for anions, such as phosphate (16). Further microarray expression experiments identified an ORF in mRNAs of *V. cholerae* expressed in response to growth on the chitin oligosaccharides, and VC0972 was identified as the *chip* gene encoding chitoporin (ChIP), which serves as a chitooligosaccharide transporter (12). The predicted gene product, referred to as VcChiP, is moderately homologous to the previously characterized chitoporins from *Vibrio furnissi* (17) and *V. campbellii* (formerly *V. harveyi*) (VhChiP) (4, 18–22). The genes encoding ChIPs are conserved in marine *Vibrio* species, emphasizing the physiological importance of the outer membrane ChIP in chitin utilization as the primary transporting system for chitinous nutrients. At the molecular level, it is still unclear how ChIP mediates the specific uptake of chitooligosaccharides, although...
our recent report suggests that the N-plug located on the periplasmic side plays an important role in substrate translocation by the ortholog VhChiP (4).

In this study, we identified the chip gene (GenBank accession no. VC0972), which encodes VcChiP, in the genome of V. cholerae. The chip fragment was cloned into the pET23d (+) expression plasmid, and the recombinant VcChiP was expressed in the outer membrane protein (omp)-deficient Escherichia coli BL21(Omp8) strain. Using electrophysiologi cal and biochemical approaches, we characterized chitooligosaccharide permeation through VcChiP reconstituted into artificial phospholipid membranes. Cell growth experiments were also carried out to verify the important role of VcChiP in the growth of the bacteria on chitooligosaccharides.

Results

Sequence analysis and structure prediction

The chip gene fragment encoding the full-length VcChiP was successfully ligated into the pET23d (+) expression vector, so as to be expressed under the control of the T7 promoter. Physical and chemical parameters for VcChiP were predicted from the ProtParam tool at the ExPASy bioinformatics resource portal (RRID:SCR_018087). The theoretical mass of the mature VcChiP lacking its signal peptide was 35,584 Da, and the predicted pI was 4.7. Here, we analyzed the sequence similarity between the newly identified VcChiP from V. cholerae and the recently characterized VhChiP from V. harveyi (18, 21, 22). As shown in the sequence alignment in Fig. 1, the amino acid sequence of VcChiP (UniProtKB entry Q9KTD0) is 38% identical to that of VhChiP (UniProtKB entry L0RVU0). The predicted secondary structure of VcChiP comprises 16 β-strands (B1–B16) flanked by 8 extracellular loops (L1–L8). There are three short α-helices, one at the N terminus and the other two on loop L3. The short helix located at the N terminus, referred to as the N-plug in VhChiP, contains 9 amino acids, whereas only 3 amino acids are predicted in VcChiP (residues LSD, in green). Long loops, other than loops L2, L3, L4, and L7, are similar in length to those in VhChiP, whereas L1 and L8 of VcChiP (residues in green) appear to be longer than those in VcChiP. In contrast, loop L5 of VcChiP (residues in red) is longer than L5 of VhChiP.

The modelled structure of VcChiP was analyzed further. Figure 2A shows a top view of the homotrimeric subunits of VcChiP. One of the three subunits is depicted by a surface representation, showing the overall electrostatic features of the internal and extracellular surfaces of the protein channel to be strongly negatively charged, based on the ABPS electrostatics scale (Fig. 2A). The other two subunits are cartoon representations, showing the β-barrel assemblies, like those of all known porins. The most prominent loop, known as L3, consists of two short helices protruding toward the central part of the channel lumen. This loop has been suggested to control the size of molecules that pass through the protein cylinder, and as such it has been referred to as the pore constriction loop (4, 23–26). Figure 2B is a superimposition of the modelled structure of VcChiP on the crystal structure of VhChiP, yielding an r.m.s.d. of 0.147 Å for 290 Cα atoms, a value that indicates high similarity in their overall structures. Loop L3, residing within the lumen (Fig. 2B, cyan for VhChiP and orange for VcChiP), is essentially identical in the two ChiPs. We previously showed that residue Trp136 in loop L3 of VhChiP played an important role in regulating sugar binding and permeation (19). The sequence alignment identified Trp118 in L3 of VcChiP in a location equivalent to that of Trp136 of VhChiP (Fig. 1, red, open box). Three loops that are noticeably dissimilar in the two ChiPs are L1, L5, and L8. Superimposition of the Cα traces clearly shows that loop L5 is highly mobile and extraordinarily long in VcChiP but short in VhChiP (Fig. 2B, red). On the other hand, loops L1 and L8 are much longer in VhChiP than the corresponding loops in VcChiP (Fig. 2B, cyan). A short helix is located at the N terminus of both proteins but is much shorter (3 amino acids) in VcChiP than in VhChiP (9 amino acids).

Gene cloning and expression of recombinant VcChiP in E. coli

The chip gene was cloned into the pET23d (+) expression vector, ready to be expressed in the omp-deficient E. coli BL21 (DE3) Omp8 Rosetta cell. The recombinant VcChiP, with its own 23-amino-acid signal sequence, was successfully expressed and inserted into the cell wall of the E. coli host. VcChiP was extracted from the cell walls with 2% (w/v) SDS, followed by 3.5% (v/v) octyl–n-octylypolyoxyethylene. The purity of the protein obtained after detergent extraction was 80–90%, and it was further purified by ion exchange chromatography on a HiTrap Q HP column, followed by gel filtration on a HiPrep® 16/60 Sephacryl S-200 column. The purity after two chromatographic steps was 95–99%, with a final yield of 5–10 mg protein per liter of bacterial culture. The purified VcChiP was subjected to trypsin digestion, and the resultant tryptic peptides were analyzed by MALDI-TOF MS before functional characterization was carried out. Nine tryptic peptides with monoisotopic masses compatible with the calculated masses were clearly identified (Table 1) in the putative sequence of VcChiP, providing 41% sequence coverage. The result confirmed that the recombinant protein expressed in the E. coli BL21 (DE3) Omp8 host was VcChiP.

Single-channel properties of the VcChiP channel reconstituted in phospholipid membranes

Pore-forming activity of VcChiP was examined at the single-molecule level, using the black lipid membrane (BLM) reconstitution technique. With protein addition on either the cis or the trans side of the phospholipid membrane, reconstituted VcChiP induced a sudden jump of ion current from zero to higher levels under applied potentials, indicating that the recombinant VcChiP could form channels, allowing ion flow and detectable ionic currents. However, the VcChiP channel exhibited significantly different behavior from that of the previously studied VhChiP (22), despite the overall resemblance in their structures, in the following respects. (1) The ionic current passing through the VcChiP channel was rapidly fluctuating, with spontaneous gating throughout the recording time (Fig. 3). (2) The VcChiP channel clearly exhibited voltage dependence, being rarely open at low positive potentials (Fig. 3, A and
B, respectively) but more regularly open and fluctuating at the equivalent negative potentials (Fig. 3, E and F). Frequent channel openings were induced by applying high, physiologically irrelevant potentials, i.e. > ±150 mV (Fig. 3, C and D and G and H) (3). The channel responded differently to positive and negative applied potentials. The ionic currents were significantly greater at negative potentials than at the corresponding positive potentials, yielding higher conductance values of the protein subunits at negative than at positive potentials. (4) With protein addition on the cis side, positive potentials additionally induced protein gating, with the opening of substate O1′ (Fig. 3, A and B) and substates O′1 and O′2 (Fig. 3, C and D,
histogram analysis) between the fully open states $O_1$ and $O_2$, respectively. The open states $O_2$ and $O_3$ were rarely observed.

(5) Much less gating was observed with negative potentials. Opening of mono-, di-, and trimeric subunits to the fully open states $O_1$, $O_2$, and $O_3$ was observed (Fig. 3, $E$–$H$, histogram analysis). The trimeric openings were seen more regularly at applied potentials above $150$ mV (Fig. 3, $G$ and $H$, respectively).

The opposite results were obtained with protein addition on the trans side. Figure 4 shows representative ion traces for continuous recording times of 1 min at different applied potentials. Figure 4, $A$–$D$, shows that ion currents were more stably open, with fully open states $O_1$, $O_2$, and $O_3$ having discrete increases in potential from $+50$ to $+100$, $+150$, and $+199$ mV, respectively. In marked contrast, ion currents at negative potentials were highly fluctuating, with additional substates $O_1^{+}$, $O_2^{+}$, and

| Peptide no. (Position in the sequence) | Expected mass (m/z) | Observed mass (m/z) | Peptide sequence |
|---------------------------------------|---------------------|---------------------|------------------|
| P1 (72–93)                            | 2494.09             | 2493.08             | DMFDNVNVFMQIESGYVEDGK |
| P2 (101–112)                          | 1336.65             | 1335.65             | DTFLGLQGDWGBK     |
| P3 (118–137)                          | 2321.15             | 2320.16             | MLTPLYEIVDWPSPNPLGR |
| P4 (138–152)                          | 1721.79             | 1720.8              | VFDWGGDVAGHYDRK   |
| P5 (158–174)                          | 1772.85             | 1771.85             | YDSPAFGGLTFNISAGR |
| P6 (264–286)                          | 2629.24             | 2628.2              | GQGEQGQGSLLQYWNPWFK |
| P7 (304–317)                          | 1625.69             | 1624.77             | DDDEVLSAQLMYVK    |
| P8 (318–325)                          | 965.52              | 964.51              | NGFVPYIR          |
| P9 (326–338)                          | 1433.67             | 1432.66             | VGQHDAYDSADKK     |

Figure 3. Single-channel recordings of VcChiP added on the cis side. Current traces (1000 ms) were obtained from single VcChiP pores at various applied potentials. Lipid bilayers were formed across a 70-μm aperture by the lowering and raising technique, using 5 mg ml $^{-1}$ 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC) in n-pentane and with 1 M KCl–20 mW HEPES, pH 7.5, on both sides of the chamber. Ion current fluctuations at four different positive potentials are shown ($A$–$D$, left) with the corresponding histograms ($A$–$D$, right) and at four different negative potentials ($E$–$H$, left) with the corresponding histograms ($E$–$H$, right).
O₃’ between the main open states O₁, O₂, and O₃ (Fig. 4, E–H). Larger ion currents were seen with positive potentials in the case of trans addition. At +199 mV, three subunits were almost constantly open (Fig. 4D), whereas −199 mV generated flickering currents (Fig. 4H).

The ion current-voltage (I-V) relationship was further analyzed. Figure 5 shows plots of ion currents versus applied potential over the range of −199 to +199 mV, with protein addition on the cis (Fig. 5A) and trans (Fig. 5B) sides. The asymmetrical behavior of the channel response to positive and negative values of the applied potential is clear. The I-V plots show that with cis-side addition (Fig. 5A), the channels were more fully open at negative potentials with the main opening states O₁, O₂, and O₃. Positive potentials generated highly gated openings with minor substates O’₁ and O’₂. The results were reversed with trans-side addition (Fig. 5B). Single-channel recordings were carried out multiple times to obtain a reliable population of VcChiP insertions (n = 33). It was consistently found that the channel had greater conductance at negative potentials than at positive potentials with addition on the cis side (Fig. 5C). Among all these measurements, we observed only one insertion showing the opposite behavior, with larger conductance at positive potentials and channel gating with lower subconductance values at negative potentials. These results indicate the asymmetric insertion of VcChiP into lipid membranes. Again, with protein added to the trans chamber, the conductance values were larger with positive potentials than with negative ones (Fig. 5D), and the conductance values increased in a nonproportional fashion as the applied potential was increased in either direction. The effects of voltage on conductance values were most pronounced with negative potentials for cis-side addition and with positive potentials for trans-side addition.

Table 2 summarizes the representative conductance values of subunit openings at ±175 mV for both cis- and trans-side addition. Considering the cis/−175 mV condition, the conductance values for mono-, di-, and trimeric subunits (fully open states O₁, O₂, and O₃) are 0.6 ± 0.1, 1.3 ± 0.2, and 1.9 ± 0.2 nS. For the cis/+175 mV condition, full trimeric opening scarcely occurred, so it was impossible to determine the conductance value for the state O₃. The conductance values for full conductance of states O₁ (0.4 ± 0.01 nS) and O₂ (0.8 ± 0.04 nS) are approximately two-thirds of the corresponding values under negative potential. Subconductance values for states O’₁ and O’₂ additionally are estimated at 0.2 ± 0.02 and 0.6 ± 0.01 nS, respectively. Regarding the trans/+175 mV condition, the conductance values for the fully open states O₁, O₂, and O₃ are
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Figure 5. I-V curves of VcChiP and voltage dependence of open channel conductance. The top panel is the I-V curve of VcChiP at potentials of ±25 to ±199 mV, with protein addition on either the cis or trans side (A and B, respectively). The data are mean values from three independent pores, and the bars show standard deviations. O₁–O₃ are the current fluctuations occurring through the fully open subunits, and O₄–O₆ are the current fluctuations occurring through substates of these subunits. The bottom panel shows the voltage dependence of open-channel conductance (C for cis side and D for trans side).

Table 2

| Opening state/substate | Channel conductance (G; nS) |
|-----------------------|----------------------------|
|                       | cis                        |
|                       | +175 mV -175 mV            |
| O₁                   | 0.2 ± 0.02                 |
| O₂                   | 0.4 ± 0.01                 |
| O₃                   | 0.6 ± 0.01                 |
| O₄                   | 0.8 ± 0.04                 |
| O₅                   | 1.3 ± 0.2                  |
| O₆                   | 1.9 ± 0.2                  |

|                       | trans                      |
|                       | +175 mV -175 mV            |
| O₁                   | 0.3 ± 0.01                 |
| O₂                   | 0.4 ± 0.01                 |
| O₃                   | 0.7 ± 0.02                 |
| O₄                   | 1.3 ± 0.01                 |
| O₅                   | 1.1 ± 0.01                 |
| O₆                   | 1.4 ± 0.06                 |

The electrolyte was 1 M KCl–20 mM HEPES, pH 7.5. Ion currents were recorded for at least 2 min using an Axopatch 200B amplifier (Molecular Devices) in the voltage-clamp mode with the internal filter set at 10 kHz. The data were analyzed by pClampfit v. 9.4.26. The conductance values were estimated from at least three separate insertions.

roughly the same as those under the cis/+175 mV condition. Notably, the conductance values for dimeric (1.0 ± 0.01 nS) and trimeric (1.4 ± 0.06 nS) subunits from the trans/-175 mV condition are significantly less than the values from the trans/+175 mV condition. The subconduction values for the subunits O₁′, O₂′, and O₃′ are 0.4 ± 0.01, 0.7 ± 0.02, and 1.1 ± 0.01 nS, respectively. The values for O₁′ and O₂′ are slightly larger than the corresponding values under the cis/+175 mV condition.

Interaction of single VcChiP channels with chitooligosaccharides

The previously characterized VhChiP exhibited strong binding affinity and permeability to long-chain chitooligosaccharides, with chitohexaose being the preferred substrate (4). We further examined the response of VcChiP to chitohexaose as a representative chitooligosaccharide. Single channels of VcChiP were reconstituted into phospholipid membranes in BLM, and ion flow was recorded with different applied concentrations of chitohexaose (Fig. 6). Because of the spontaneous gating of the VcChiP channel in phospholipid membranes, it was difficult to distinguish sugar-induced blocking events from channel gating under high applied potentials. In this set of experiments, we selected the cis/trans and ±50 mV conditions for this study, as the ionic currents under low potentials were relatively small. Figure 6, A–D, shows representative traces recorded at −50 mV with the protein and sugar additions on the cis side. The ion trace in the absence of sugar (Fig. 6A) showed monomeric and dimeric openings and closings between O₁ and O₂ states. When the channel was exposed to chitohexaose, we generally observed increases in conductance values in a concentration-dependent manner. The dimeric and trimeric conductance levels are noticeable when concentrations of chitohexaose were increased from 10 (Fig. 6B) to 50 mV (Fig. 6C) and 40 μM (Fig. 6D), respectively. In contrast, stable current flow was seen with the homologous VhChiP channel (Fig. 6E), allowing the blocking events to be well resolved after sugar addition. It was noticeable that the VhChiP channel was much more responsive to chitohexaose, as dimeric and trimeric blockades were observed at much lower concentrations of the sugar, i.e. 1.25 μM (Fig. 6F) to 2.5 μM (Fig. 6G) and 5.0 μM (Fig. 6H). The sugar-induced conductance changes observed for VcChiP were not observed with VhChiP.

Sugar permeability into VcChiP-reconstituted proteoliposomes

Purified VcChiP was reconstituted into proteoliposomes, and bulk permeation of various sugar species through VcChiP was examined in vitro by liposome swelling assays. In this assay,
the permeation rate of the smallest sugar (\(\text{\text{-arabinose, } M_r 150.1}\) was set to 100%. A nonpermeant branched sugar (raffinose, \(M_r 504.4\)) was used to determine the isotonic concentration of the prepared proteoliposomes. The permeation rates of the small sugars that entered the proteoliposomes through \(\text{VcChiP}\) were compared with that of the smallest sugar (\(-arabinose). The results shown in Fig. 7A suggested that all monosaccharides (\(-glucose, \(-mannose \(-galactose, and \text{GlcNAc}) could permeate through both \(\text{VcChiP}\) and \(\text{VhChiP}\) at similar rates (70–75%). However, disaccharides (maltose, \(M_r 360\), and sucrose, \(M_r 342\)) could not permeate through \(\text{VcChiP}\) at all, whereas they permeated through \(\text{VhChiP}\) at very low rates (<10%). The trisaccharide melezitose was impermeant through both ChiPs. Figure 7B shows that chitosaccharides (\text{GlcNAc, chitobiose, chitotriose, chitopentaose, and chitohexaose}) could permeate into the \(\text{VcChiP}\)-reconstituted proteoliposomes at comparable rates (~16–25% permeability), whereas other hexose sugars, including chitosan hexamer, maltohexaose, and cellohexaose, were impermeant.

**Effects of various carbon sources on the growth of the omp-deficient \(\text{E. coli BL21(DE3)}\) Omp8 host expressing \(\text{VcChiP}\)**

The growth of \(\text{E. coli Omp8}\) host carrying \(\text{VcChiP}\) on M9 minimal medium supplemented with various carbon sources was monitored. As shown in Fig. 8, \(\text{E. coli Omp8}\) cells did not grow on minimal medium without any supplementary carbon source, regardless of \(\text{VcChiP}\) expression (Fig. 8, \(A–F\), symbols ■, ▲, and ○), whereas \(\text{E. coli Omp8}\) cells grew well on glucose, glycerol, or \text{GlcNAc} supplements, with or without heterologous \(\text{VcChiP}\) expression (Fig. 8, \(A–C\), symbols □, △, and ○, respectively). The highest growth rate was observed with \(\text{E. coli Omp8}\) with no \text{chip} gene, grown with a \text{GlcNAc} supplement (Fig. 8C, symbol □). Figure 8D shows that the growth of \(\text{E. coli Omp8}\) cells without expressed \(\text{VcChiP}\) was diminished with the disaccharide (\text{GlcNAc})2 supplement, with a prolonged lag phase of 35 h (Fig. 8D, symbols □ and △), whereas cells expressing \(\text{VcChiP}\) entered logarithmic phase much faster at an incubation time of 16 h (Fig. 8D, symbol ○). \(\text{E. coli Omp8}\) cells not expressing \(\text{VcChiP}\) were unable to grow on chitooligotrisaccharide (\text{GlcNAc})3 (Fig. 8E, symbols □ and △), whereas cells expressing \(\text{VcChiP}\) could grow but with a prolonged lag phase of 25 h (Fig. 8E, symbol ○). There was essentially no growth of \(\text{E. coli Omp8}\) cells on (\text{GlcNAc})4 with or without \(\text{VcChiP}\) expression, even with 60 h of incubation (Fig. 8F).

**Discussion**

Cholera infection caused by \(\text{V. cholerae}\) has been a subject of health concern, as the disease has claimed millions of human lives worldwide. \(\text{V. cholerae}\) O1 and O139 are highly infectious strains that are responsible for most incidences of endemic and epidemic transmission, as they carry genes encoding cholera toxin and the toxin coregulated pilus (27). Adhesion to chitinous surfaces is crucial for the survival of the bacteria (28), as it is a prerequisite step for nutrient recruitment (1, 7, 29–31), which enables the bacteria to thrive under harsh aquatic conditions (32). Subsequent
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**Figure 7.** Proteoliposome swelling assays. Proteoliposomes were prepared as described in Materials and methods. α-Raffinose was used to determine the isotonic concentration, which produced no change in optical density at 500 nm over 60 s, and the swelling rate of α-arabinose was set to 100%. The rate of permeation through the channel was assumed to be proportional to the swelling rate. A, Permeation of small sugars through reconstituted VcChiP and VhChiP into proteoliposomes. B, Permeation of chitooligosaccharides through VcChiP. Chitosan hexamer, maltohexaose, and cellohexaose were used as a control. Values are means ± S.D., obtained from 3 independent sets of experiments.

**Figure 8.** Growth of the omp-deficient E. coli BL21(DE3) Omp8 Rosetta strain on various carbon sources. The E. coli cells were grown on M9 minimal medium supplemented with glucose (A), glycerol (B), GlcNAc (C), (GlcNAc)2 (D), [GlcNAc]3 (E), and (GlcNAc)x (F). Symbols: ■, no added carbon source; △, E. coli Omp8 with carbon source; ▲, E. coli Omp8 carrying pET23a (+)/chip gene with no IPTG induction and no carbon source; □, E. coli Omp8 carrying pET23a (+)/chip gene with IPTG induction and with a carbon source; ●, E. coli Omp8 carrying pET23a (+)/chip gene with IPTG induction and no carbon source; ○, E. coli Omp8 carrying pET23a (+)/chip gene with IPTG induction and a carbon source. OD 600, optical density at 600 nm.

Biofilm formation also enhances resistance to acidic digestion in the human gut, enabling efficient colonization (8, 33, 34) and enhancing the virulent invasion of host organs (6, 35–37).

It has been shown that V. cholerae expresses several chitin-inducible proteins, such as chitinases, chitin-binding proteins, and transporters, that are involved in their growth on chitin surfaces (6, 38, 39). There are few detailed reports of the structures and molecular functions of these chitin-induced components in V. cholerae; however, the chitinolytic cascades in other Vibrio species are more fully elucidated. In our previous studies, we reported the functional and structural characterization of several chitin-utilizing components of the homologous species V. campbellii strain TCC BAA-1116/BB120 (formerly V. harveyi) (11, 12, 17, 40–46). A recently identified example is an outer-membrane chitoporin, VhChiP (4, 22). The crystal structures revealed that VhChiP is a pore-forming entity, comprising three β-barrel structures. VhChiP can insert into phospholipid membranes and form a stable channel with single-channel conductance of 1.8 ± 0.5 nS (18) and is highly specific for chitooligosaccharides, with chitohexaose being the preferred substrate. In this study, we report the first chitooligosaccharide-specific channel, referred to as VcChiP, in the highly infectious strain V. cholerae O1. The modelled structure suggested that VcChiP contains three identical subunits that form a typical barrel structure, like those of other known porins (23, 47, 48). Despite close similarities in the structures of VhChiP and VcChiP, their single-channel behaviors are completely different. The trimeric cylinder of VhChiP was shown to be fully open even at low voltages above ±25 mV and to remain open up to ±150 mV. In marked contrast, the VcChiP channel showed prominent gating characteristics in actively opening and shutting throughout the measurements. Nevertheless, certain gating patterns could be established, using recording times of at least 2 min with numerous insertions. Subconductance values O1’, O2’, and, occasionally, O3’, observed between the fully open states O1, O2, and O3, were dictated by the polarity of transmembrane potentials. Such behavior reflected the asymmetric insertion of the protein channel into lipid membranes. The results allowed us to select ion traces from the same insertion side throughout our study. Previous studies suggested that several outer-membrane porins tended to insert asymmetrically into planar bilayers, with the long hydrophilic loops (referred to as extracellular loops) retained on the cis side after protein addition to the cis side (48–50). However, there are some exceptions, such
as monomeric OmpG, which was reported to insert into membranes with bidirectional orientation (51).

The pronounced gating was not a feature of VhChiP, and the modeled structures of the two channels indicate clear differences in their loop regions. Loop L5 is exceptionally long in VcChiP, whereas loops L1 and L8 are more prominent in VhChiP (4). All of these loops are surface-exposed and presumably flexible. Loop L5 of VcChiP is of particular importance for further investigation, as the increased length of this loop may be responsible for the gating behavior of the VcChiP channel, which was not observed in the highly stable VhChiP channel, with a short L5. Loop L5 was also observed as an extra segment, in addition to loop L3, inside the lumen of the highly active BpsOmp38 from the melioidosis bacterium B. pseudomallei (unpublished crystal structure), whereas this loop is very short in the stably opening channels LamB (PDB entry 1MAL) and OmpF (PDB entry 2ZFG).

The VcChiP channel clearly exhibited nonohmic ion conductance and voltage dependence profiles, with greater ion flow at negative potentials with protein addition on the cis side and, conversely, when the protein was added on the trans side. With the cis side set as the ground side of our BLM setup, such flow indicates greater permeability of cations (K+ in our study) than of anions; thus, the channel is predicted to be cation-selective, like the OccD1-6 channel from the highly infectious pathogen Pseudomonas aeruginosa, identified by Liu et al. (52). The bulk permeability of different tested sugars, measured by liposome swelling assays, suggested that VcChiP allowed the passage of monosaccharides by general diffusion but exhibited a size-exclusion limit for most sugars of approx. 300 Da. This was deduced from the significantly reduced permeability of GlcNAc (M, 220 Da) and the impermeability of sucrose and maltose (M, 342). Apart from the chitooligosaccharides, larger sugars were impermeant, which is a characteristic of the chitooligosaccharide-specific porins VhChiP and EcChiP, identified previously (22, 53). Comparing the permeability to chitooligosaccharides of the two ChiP homologues, VcChiP had lower overall permeability toward all chitooligosaccharides and broader substrate specificity (18), reflecting the conditions in the natural habitats of the two Vibrio species. VhChiP is essential for V. harveyi, which lives in marine environments, where chitin is its sole carbon source. In marked contrast, VcChiP could facilitate the uptake of chitin as an alternative carbon source for V. cholerae, allowing it to survive in the plankton-bound state. Once inside the human gut, glucose is its most likely energy source, allowing the bacterium to persist in the infectious state. This assumption is supported by data from the growth of omp-deficient E. coli Omp8 cells expressing VcChiP. With no ChiP induction, the cells grew well on their natural carbon sources, including glucose and glycerol. When other carbon sources were depleted, cells with induced VcChiP could immediately use GlcNAc as the primary source. Delayed growth was seen when the cells were supplemented with (GlcNAc)₃, probably because the cells required a longer time for the metabolism of the disaccharide by inducible GlcNAcase. Greatly delayed growth was seen on (GlcNAc)₄ and no growth on (GlcNAc)₅-supplemented minimum medium under the given times of incubation, indicating the absence of active chitinase expression in the E. coli Omp8 strain. Overall, the data obtained from cell growth studies confirmed the physiological role of VcChiP in the survival of the bacteria under conditions of restricted chitin availability.

Conclusions

We have demonstrated, for the first time, a chitin transport protein in the highly infectious bacterium V. cholerae. The aim of further studies would be to reveal which structural elements of the channel control its gating, voltage dependence, and ion selectivity. Understanding the structural basis of ion transport by VcChiP would introduce the opportunity to design sugar-based molecules that could interact efficiently with the channel, whereas surface proteins of V. cholerae, including VcChiP along with other virulence factors, could serve as excellent candidates in the development of novel vaccines and antimicrobial agents to combat cholera infection.

Materials and methods

Gene cloning and DNA sequencing

The open reading frame encoding chitoporin of Vibrio cholerae O1 biovar El Tor was identified in the GenBank database (accession no. DQ774012.1), and the chip gene was synthesized in the pUC57 cloning vector using the GenScript Gene Synthesis Service (GenScript, Piscataway, NJ, USA). The pUC57/chip construct was used as the DNA template for PCR amplification of the chip gene fragment, using the BamHI restriction site for the design of the forward primer and the XhoI restriction site for the reverse primer. The oligonucleotides used for PCR amplification were 5'-ATGGATCCATGGTTGACAAATGTTT-3' for the forward primer and 5'-AACCTCGAGTCACAGAACAC-ACCGTA-3' for the reverse primer. Underlined sequences indicate the restriction sites. The DNA insert of the expected size was excised and ligated with the pGEM-T cloning vector, according to the manufacturer's instructions, with A-tailing, and then the chip gene fragment was transferred into the pET23d (+) expression vector using the BamHI and XhoI cloning sites. In the final step, the nucleotide sequences of the sense and anti-sense strands of the chip fragment were determined by automated sequencing (First BASE Laboratories SdnBhd, Selangor DarulEhsan, Malaysia).

Structure prediction and sequence analysis

The amino acid sequences of V. cholerae ChiP (UniProtKB entry Q9KTD0) and V. harveyi ChiP (UniProtKB entry L0RVU0) were aligned by ClustalW2 (RRID:SCR_002909).

The structural elements of VcChiP were constructed using the program ESPript v3.0 (RRID:SCR_006587), with the crystal structure of VhChiP (PDB entry 5MDO) as the structure template. The amino acid sequence of VcChiP was submitted to the Swiss-Model server (RRID:SCR_013032) for tertiary structure prediction using the crystal structure of VhChiP (PDB entry 5MDO) as the structural template. The annotated structure was edited and displayed in PyMOL (RRID:SCR_000305).
Expression and purification

Recombinant VcChiP was expressed in the cell walls of E. coli Omp8 cells and purified as described previously (18). Briefly, the expression vector pET23d(+), harboring the full-length chip gene, was transformed into E. coli BL21(DE3) Omp8 Rosetta strain (here referred to as E. coli Omp8). The transformed cells were grown at 37 °C in Luria-Bertani (LB) broth supplemented with 100 μg ml⁻¹ ampicillin and 25 μg ml⁻¹ kanamycin. During the exponential growth phase (optical density at 600 nm of ~0.6–0.8), heterologous VcChiP expression was induced with 0.5 mm isopropyl thio-galactoside (IPTG). After 6 h of further incubation at 37 °C, the cell pellet was harvested by centrifugation at 2948 × g for 25 min at 4 °C. For protein purification, the cell pellet was 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. Cells were disrupted with a high-speed ultrasonic homogenizer with a 6-mm-diameter probe (EmulsiFlex-C3, Avestin Europe, Mannheim, Germany) for 10 min. For cell wall extraction, SDS solution was added to the homogenized pellet (final concentration, 2% w/v), and the suspension was incubated at 50 °C for 60 min with 300 rpm shaking to ensure complete lysis. Cell wall components were removed by centrifugation at 100,000 × g at 4 °C for 1 h. The VcChiP-containing pellet was extracted twice with 3.5% (v/v) octyl-polyoxyethylene (ALEXIS Biochemicals, Lausanne, Switzerland) in 20 mM phosphate buffer, pH 7.4, and centrifuged again. The supernatant was then dialyzed thoroughly against 20 mM phosphate buffer, pH 7.4, containing 0.05% (v/v) lauryldimethylamine oxide (Sigma-Aldrich Pte. Ltd., Singapore). The solubilized VcChiP was subjected to ion-exchange chromatography using a Hitrap Q HP prepacked column (5 by 1 ml) connected to an ÄKTA Prime plus FPLC system (GE Healthcare Life Sciences, Life Sciences Instruments, ITIS [Thailand] Co. Ltd., Bangkok, Thailand). Bound proteins were eluted with a linear gradient of 0–1 M KCl in 20 mM phosphate buffer, pH 7.4, containing 0.05% (v/v) lauryldimethylamine oxide. The purity of the eluted fractions was confirmed by SDS-PAGE. To obtain protein of high purity, fractions containing VcChiP were pooled and subjected to size exclusion chromatography using a HiPrep 16/60 Sephacryl S-200 high-resolution column. The protein concentration of the freshly prepared VcChiP was estimated using the Novagen BCA protein assay kit (EMD Chemicals Inc., San Diego, CA, USA), and the identity of the purified VcChiP was confirmed by MALDI-TOF MS analysis (BGI Tech Solutions [Hong Kong] Co. Ltd., Hong Kong).

Single-channel recording experiments

Solvent-free bilayer (Montal-Mueller type) formation was performed using 5 mg ml⁻¹ 1,2-diphtyanoxy-sn-glycero-3-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) in n-pentane. First, a 25-μm-thick Teflon film with an aperture of 40–70 μm was sandwiched between the two chambers (designated cis and trans) of a cuvette, and the aperture was prewetted with 1–2 μl of 1% (w/v) hexadecane in n-hexane. Both chambers were filled with electrolyte (1 M KCl–20 mM HEPES, pH 7.5), and BLM experiments were carried out at room temperature (25 °C). A planar lipid bilayer was formed across the aperture by lowering and raising the liquid level (54). Ionic currents were detected using Ag/AgCl electrodes, one connected to the cis (ground) side of the membrane and the other (trans) to the head stage of the Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Single-channel measurements were performed in the voltage clamp mode and digitized using the Axon Digidata 1550 digitizer, and data acquisition was performed using Clampfit software (Axon Instruments). The traces obtained were filtered at 10 kHz, using a low-pass Bessel filter with a sampling frequency of 50 kHz. Single-channel analyses were performed using Clampfit software (Molecular Devices, Sunnyvale, CA, USA). Single protein channels were reconstituted in the lipid bilayer by adding VcChiP (10–100 ng) to either the cis- or the trans-side of the cuvette. Conductance values were extracted from the current steps observed at different voltages after the addition of the protein using the I-V curve. VcChiP was titrated with discrete concentrations of chitooligosaccharides of various chain lengths, added to the cis side of the chamber. Fluctuations of ion flow observed as a result of sugar diffusion through the inserted channel were usually recorded for 2 min at different transmembrane potentials (±25 to ±199 mV).

Growth of E. coli BL21(DE3) Omp8 Rosetta expressing heterologous VcChiP on various carbon sources

To test the effects of chitoporin expression on the utilization of various carbon sources, E. coli Omp8 cells with heterologous VcChiP expression were grown in a microtiter plate containing 200 μl of M9 minimal medium supplemented with various carbon sources, and growth was monitored with a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Ltd., Bangkok, Thailand). The protocol used for this experiment was slightly modified from a previous report (55). E. coli cultures were first grown aerobically at 37 °C in LB medium supplemented with 1% (w/v) d-glucose, and VcChiP expression was induced by adding 0.5 mM IPTG. Cells were harvested by centrifugation and resuspended to the desired starting optical density at 600 nm in the appropriate fresh M9 minimal medium. Inoculated cultures were grown aerobically in an Eppendorf ThermoMixer® C (350 rpm) in a microtiter plate containing 200 μl of M9 minimal medium supplemented with various carbon sources, all at 0.20% (w/v): d-glucose, glycerol, GlcNAc, GlcNAc₂, or GlcNAc₃, E. coli Omp8 with no plasmid and E. coli Omp8 carrying the pET23d(+)VcChiP plasmid but not expressing ChiP (no IPTG addition) were used as controls.

Proteoliposome swelling assays

Proteoliposomes were prepared by following the protocols described elsewhere (22, 56). Soybean l-α-phosphatidylcholine (20 mg ml⁻¹, freshly prepared in chloroform) (Sigma-Aldrich [Thailand] Co. Ltd., Bangkok, Thailand) was used to form multilamellar liposomes. For the preparation of proteoliposomes, 200 ng of VcChiP was reconstituted into the liposomes by sonication and dried in a desiccator overnight. The dextran-filled
liposomes were prepared by resuspending the dried proteoliposomes in 5 mM sodium phosphate buffer, pH 7.5, containing 17% (w/v) dextran from *Leuconostoc* spp. (M, 40,000) (Sigma-Aldrich). The isotonic concentration was determined with different concentrations of d-raffinose (50, 60, 70, and 80 mM) prepared in 5 mM sodium phosphate buffer, pH 7.5, and the value obtained was used to make isotonic solutions for other sugars. To perform the liposome swelling assay, 25 µl of the proteoliposome suspension was added to 600 µl of sugar solution, and changes in absorbance at 500 nm were monitored immediately for 60 s using a T80+ UV-Vis spectrophotometer (PG Instrument Ltd., UK). The apparent absorbance change over the first 60 s was used to estimate the swelling rate (s⁻¹) according to the equation $\phi = (1/A_i)da/dt$, where $\phi$ is the swelling rate, $A_i$ the initial absorbance, and $da/dt$ the rate of absorbance change over the first 60 s. The sugars tested were d-glucose, d-mannose, and d-galactose (M, 180), GlcNAc (d-GlcNAc, M, 221), d-sucrose (M, 342), d-melezitose (M, 522), chitobiose ([GlcNAc]₂, M, 424), chitotriose ([GlcNAc]₃, M, 628), chitotetraose ([GlcNAc]₄, M, 830), chitopentaose ([GlcNAc]₅, M, 1034), chitohexaose ([GlcNAc]₆, M, 1237), chitosan hexamer (M, 1204), maltotriose (M, 991), and cellobiose (M, 991). The permeation rate for each sugar was normalized by setting the rate for l-arabinose (M, 150) to 100%. The presented values are means from three independent determinations. Protein-free liposomes and proteoliposomes without sugars were used as negative controls.

**Data availability**

Raw data for mass identification are available online from Zenodo (3760230). All other data used for this publication are to be shared upon request. Please contact wipa.s@vistec.ac.th and sasimalisoysoya@gmail.com.

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**Conflict of interest**—All authors declare that they have no competing financial interest.

**Abbreviations**—The abbreviations used are: ChiP, chitoporin; omp, outer membrane protein; IPTG, isopropyl thio-β-d-galactoside; BLM, black lipid membrane.

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