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The Outer Membrane Protein VhOmp of *Vibrio harveyi*: Pore-Forming Properties in Black Lipid Membranes

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Abstract *Vibrio harveyi* is known to cause fatal *vibriosis* in marine animals. Here, an outer membrane protein from *V. harveyi*, namely, VhOmp, was isolated and functionally characterized in terms of pore-forming contact with artificial lipid membranes. The native VhOmp exists as a trimer of a molecular weight similar to that of the porin OmpF from *Escherichia coli*. Reconstitution of VhOmp into black lipid membranes demonstrated its ability to form ion channels. The average pore conductance of VhOmp was revealed to be about 0.9 and 2 nS in 0.2 and 1 M KCl, respectively. Within transmembrane potentials of ±100 mV, VhOmp pores behaved as ohmic conduits, and their conductance scaled linearly with voltage. Nonlinear plots of the pore conductance versus symmetrical salt concentrations at either side of the protein-incorporating membrane suggested the influence of interior channel functionalities on the passage of charged species. In the presence of Omp-specific polyclonal antibodies, the pore-forming property of VhOmp was modulated so that the usual step-like current increments were replaced by random transitory current fluctuations. VhOmp exhibited a strong biological activity by causing hemolysis of human red blood cells, indicating that VhOmp may act as a crucial determinant during bacterial infection to animal host cells.

Keywords *Vibrio harveyi* · Vibriosis · Porin channels · Outer membrane proteins · Lipid bilayer membranes · Pore conductance

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Vibrio *harveyi*, formerly referred to as *V. carchariae*, is a Gram-negative marine bacterium and the origin of the disease luminous (or luminescent) vibriosis. This infection can be fatal for sea fish, crustaceans, and mollusks and is a serious threat, in particular, for the farmed species within the multi-billion-dollar global seafood industry (Owens and Busico-Salcedo 2006; Austin and Zhang 2006; Thompson et al. 2004; Zhang and Austin 2000; Kraxberger-Beatty et al. 1990). Application of antibiotics is the routinely used preventive measure against *V. harveyi*-associated fatalities in seafood monocultures. Obviously, this practice is controversial, as antibiotic resistance may develop rapidly and there is a health risk for those who consume a probably drug-contaminated end product. Alternative strategies are therefore sought, and recently outer membrane proteins (Omps) from *V. harveyi* have been introduced as potential immunostimulants and protection against the bacterium's impact on target organisms (Arijo et al. 2008; Ninqiu et al. 2008; Qin et al. 2007; Zhang et al. 2007).

Their promising role as an effective immunogen is, however, not the only reason that Omps from *V. harveyi* are worth looking at. Omps are central for bacterial cell function. Well-studied examples of pore-forming Omps (so-called "porins") include *Escherichia coli* OmpF, OmpC, PhoE, and LamB, all of which are β barrel proteins that cross the cellular lipid membrane and act as channels controlling the traffic of chemicals into the microorganism's cytoplasm (Nikaido 2003). An adaptation of membrane permeability via evolutionary structural mutations and lowered expression levels of Omps is accepted as a strategic action that bacteria use to establish antibiotic resistance by down-regulating the flux of drug molecules into the cell interior and by restricting the access of active agents to intracellular target sites (Pagès et al. 2008; Delcour 2008; Ceccarelli and Ruggerone 2008; Kumar and

Schweizer 2005; Nikaido 2003; Poole 2002). Accordingly, a detailed analysis of the movement of nutrients, metabolic products, and, most of all, drugs through individual bacterial porins is fundamental to the understanding of the antibiotic resistance mechanism and the pathogenesis of bacterial diseases.

High time and current resolution measurements with functional Omps implemented in artificial planar lipid bilayers (also referred to as black lipid membranes; BLMs) are among the electrophysiological and biophysical assays sensitive enough for direct in situ permeability studies on the level of single porin channels (Weingart et al. 2008; Tien and Ottova 2001). BLM measurements together with other complementary techniques have been performed on porin channels from a range of bacteria, but not yet with *V. harveyi* Omps. Membrane isolates from *V. harveyi* may, however, be an alternative model system for principal investigations of the role of Omps in both bacterial membrane transport phenomena and induction of an immune response. The species is, for instance, not pathogenic to humans, and laboratory personnel would be at considerably lower health risk working with this rather than other more dangerous bacteria during fundamental studies. Moreover, the results of detailed structure/function studies with wild-type and genetically engineered variants of *V. harveyi* Omps may deliver implications for the development of effective antibiotics, and tailored immunogenic Omp proteins that may be instrumental in developing future subunit vaccines.

In this study, we isolated and functionally characterized a specific Omp from the outer membranes of *V. harveyi* cells, which is subsequently referred to as VhOmp. VhOmp in the form of trimers was successfully reconstituted into painted lipid bilayer membranes, thus allowing basic channel properties to be measured via the BLM technique. Comparative trials were performed with VhOmp as a novel porin and *E. coli* OmpF as a well-characterized reference porin. Measurements resolving the insertion of individual VhOmp trimers confirmed their pore-forming character and revealed conductance values under various electrolyte conditions and the ability to effectively induce red blood cell lysis was a clear sign of their biological activity.

Materials and Methods

Bacterial Strains and Chemicals

The marine bacterium *V. harveyi* type strain 650 was a gift from Dr. Peter Robertson, Department of Biological Sciences, Heriot Watt University, Edinburgh, UK. The *E. coli* type strain BL21 (DE3)omp8 (phenotype BL21[DE3] ΔlamB ompF::Tn5 ΔompA ΔompC [Prilipov et al. 1998]) was kindly provided by Prof. Mathias Winterhalter and Dr. Helge Weingart, Jacobs University Bremen, Bremen, Germany. Antisera against a particular Omp from *Burkholderia pseudomallei* (BpsOmp) were prepared as reported previously (Siritapetawee et al. 2004a).

All chemicals and reagents were of analytical grade and from the following sources: bacterial media, from Scharlau Chemie S.A. (Barcelona, Spain); dithiothreitol, iodoacetamide, and ammonium hydrogen carbonate from Acros Organics (Morris Plains, NJ, USA); trypsin (sequencing grade), from Promega Corporation (Bio-Active Co., Ltd, Bangkok, Thailand); *n*-octylpolyoxyethylene (octyl-POE) from Bachem A.G. (Bubendorf, Switzerland); and azolectin (2-diacyl-*sn*-glyero-3-phosphocholine; Type II-S), from Sigma-Aldrich (St. Louis, MO, USA). Reagents for general laboratory use were from Sigma-Aldrich, Acros Organics, Scharlau Chemie S.A., or Carlo Erba Reagenti (Milan, Italy).

Preparation of VhOmp from *V. harveyi* and OmpF from *E. coli*

An overnight culture (10 ml marine medium 2216E, 30°C) from a single colony of *V. harveyi* type strain 650 was used to inoculate two flasks, each containing 1000 ml of the marine medium. After about 6 h of incubation at 30°C, cells were collected by centrifugation (2,800*g*, 15 min, 4°C). After that, VhOmp was isolated following the published procedure for the isolation of *E. coli* OmpF (Garavito and Rosenbusch 1986). In the lysis step, the cell pellet (1 g) was resuspended in 10 ml of lysis buffer (20 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mg lysozyme). Cells were further sonicated using a Sonopuls ultrasonic sonicator using a 6-mm-diameter probe (50% duty cycle, 20% amplitude setting, 5-min total time), then 1 ml of 20% (w/v) SDS was added per 10 ml of cell suspension. After incubation for 1 h at 60°C with gentle shaking and centrifugation at 35,500*g* and 4°C for 1 h, the pellet containing the membrane fraction was washed once with 20 mM phosphate buffer, pH 7.4, to remove residual SDS. The pre-extraction step was performed by adding 5 ml of 0.125% (v/v) *n*-octylpolyoxyethylene (octyl-POE; prepared in 20 mM phosphate buffer, pH 7.4) to 1 g of the membrane pellet. The pellet was homogenized using a Potter–Elvehjem homogenizer and incubated for 1 h at 37°C with gentle shaking. After centrifugation at 117,300*g* at 20°C for 40 min, the supernatant containing solubilized VhOmp was collected. Further extraction was carried out by resuspending the remaining pellet obtained after previous centrifugation with 1% (v/v) octyl-POE. The pellet was homogenized using a Potter–Elvehjem homogenizer and incubated for 1 h at 37°C with shaking. The

Omp fraction was obtained in supernatant after re-centrifugation at 117,300*g* and 20°C for 40 min.

For OmpF preparation, the overnight culture of the *E. coli* BL21 (DE3)omp8 strain (10 ml; grown in LB containing 100 µg/ml ampicillin [Amp] at 37°C) was transferred to two flasks of 1000 ml LB/Amp broth. Incubation was carried out at 37°C in a shaking incubator until an OD_{600} of approx. 0.6 was reached. At this point, isopropyl β-D-thiogalactoside (IPTG) was added to the culture medium to a final concentration of 0.4 mM. Incubation was continued for an additional 90 min, then cells were harvested by centrifugation at 10,000*g* for 30 min. Further isolation of *E. coli* OmpF was performed as described for VhOmp.

Determination of Protein Concentration

Protein concentrations were estimated using the BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A protein sample (12.5 µl) was mixed with 100 µl of the BCA working reagent. After the reaction mixture was incubated at 37°C for 30 min, absorbance at 540 nm was measured with a microplate reader spectrophotometer (Labsystem, Finland). BSA at various concentrations, ranging from 0.025 to 1.0 mg/ml, was used to construct a standard calibration curve and to determine protein concentrations of unknown samples.

SDS-PAGE Following Immunological Analysis

An immunological analysis was carried out with antisera against BpsOmp from *B. pseudomallei*. The purified *Vh*Omp (3 µg) was electrophoresed on a 12% (wv) SDS-PAGE gel following a Laemmli (1970) buffer system, then transferred onto a nitrocellulose membrane using a Trans-Blot Semi-Dry Cell (BioRad, Hercules, CA, USA). Immunodetection was carried out using enhanced chemiluminescence (ECL; Amersham Biosciences) according to the manufacturer's instructions. The primary antibodies were polyclonal anti-Omp (1:2000 dilution) and the secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (1:2500 dilution).

Protein Identification and Peptide Mass Analysis by MALDI/TOF MS

The protein bands from SDS gels (see above) were excised, destained, reduced, alkylated with iodoacetamide, and digested with sequencing-grade trypsin (Promega) following a standard protocol (Shevchenko 1996). After overnight digestion at 37°C, the peptides were extracted and dried in a SpeedVac vacuum centrifuge. Tryptic peptides were sent to BioServiceUnit (BSU; Thailand Science Park, Bangkok) for matrix-assisted desorption ionization/time-of-flight mass spectrometry (MALDI/TOF MS) analysis. Databank searching was performed with MASCOT (http://www.matrixscience.com/) for peptide mass fingerprint data.

Planar Lipid Bilayer Reconstitution Experiments and Pore Conductance Analysis

The BLM setup included a patch-clamp amplifier with a two-electrode bilayer headstage (PC-ONE plus PC-ONE-50; Dagan Corp., Minneapolis, MN, USA), a Faraday cage placed on a vibration-dampening table, an A/D converter (LIH 1600; HEKA Elektronik, Lambrecht, Germany), and the software for computer-controlled operation (PULSE program; HEKA Elektronik). Support for BLMs was a 200-µm hole in a 1.5-ml Delrin cup that fit tightly into one of the two wells of a polymer bilayer chamber. The interior of the cup (*cis*) and the vacant well (*trans*) were filled with the electrolyte solution, into which the two Ag/AgCl/1M KCl reference electrodes at the amplifiers headstage were immersed. Routinely the "*cis*" electrode was voltage-clamped with respect to the "*trans*" one, which hence was coupled to signal ground. Azolectin BLMs were formed by painting a 50 mg/ml hexane solution of the lipid over a cup aperture that had been treated with a few microliters of hexadecane/hexane (1:100, v/v) and allowing it to dry. BLMs for Omp experiments had to have about 100 pF in capacitance tests and a stable, virtually leak-free current signal throughout minute-long recordings at constant potentials. Omp membrane insertion was induced by adding to the 1-ml cup (*cis*) electrolyte microliter aliquots of a stock solution that had the proteins solubilized at 100 µg/ml in 20 mM phosphate buffer, pH 7.4, and with 1% (v/v) octyl-POE. Membrane current (I_m) recordings were conducted at room temperature (25°C) with the potential across the BLM being kept at user-defined values within ±150 mV, and the acquired data filtered by a three-pole, low-pass Bessel filter at 1 kHz and saved into the computer memory with a 1-ms (1 kHz) sampling interval. Electrolytes on both sides of BLMs were symmetrical in concentration and either potassium (KCl), lithium (LiCl), and cesium (CsCl) chloride or potassium acetate (Kac) solutions in ultrapure water. Membrane activity in terms of current flow was directly analyzed via the acquisition software PULSE or stored traces were handled with Microsoft Office Excel 2007 and GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA).

Red Blood Cell Lysis Assay

Human red blood cells (RBCs) were obtained from two healthy donors using heparin as an anticoagulant. In the

first step, white blood cells and platelets were removed by Ficoll-Hypaque density gradient centrifugation. Then 150 µl of the remaining packed RBCs was added to 10 ml of PBS. The cell suspension was gently mixed and centrifuged at 400*g* for 5 min at 25°C. The procedure was repeated twice. A three percent of RBC suspension was prepared by adding 4.85 ml of PBS to the remaining cells. The RBC lysis assay was carried out in 1.5-ml microtubes following a protocol adapted from the work of others (Tramontini et al. 2002; Wang et al. 2007). Briefly, 5 µl of the 3% RBC suspension was added to 100 µl of PBS containing 0.8, 2.6, 3.1, 6.3, 12.5, 25, or 50 µg/ml VhOmp or OmpF. The solution was gently mixed and incubated at 25°C for 5 min. Complete or partial clearance of the cell suspension, indicating hemolysis of RBCs, was determined by measuring the decrease in optical density of the cell suspension at 600 nm (OD_{600}). Hemolysis of RBCs induced by distilled water was used as the positive control, and PBS as the negative control.

Results and Discussion

Isolation of VhOmp and Protein Identification

Outer membrane proteins are known to be tightly associated with the peptidoglycan layer of Gram-negative bacteria. In this study, an outer membrane protein of *V. harveyi* (later designated VhOmp) was isolated from crude peptidoglycan prepared in 20% (w/v) SDS. The outer membrane protein was pre-extracted with 0.125% (v/v) octyl-POE and further extracted with 1% (v/v) octyl-POE (see "Materials and Methods" section). As verified by SDS-PAGE (Fig. 1a), the final fraction contained mainly VhOmp that was solubilized well in 1% (v/v) octyl-POE. When the mixture was heated to 100°C for 5 min, a single band corresponding to the extracted VhOmp was seen between 30 and 45 kDa. This protein band migrated to the same position as the *E. coli* Omp (OmpF) and *B. peudomallei* Omp (BpsOmp) bands. Under unheated conditions, the three proteins were retarded on the polyacrylamide gel to positions between 66 and 97 kDa. This indicated that VhOmp, as well as OmpF and BpsOmp, was stable to SDS but sensitive to heat treatment. It is well known that OmpF and BpsOmp are trimeric structures with a molecular weight (MW) of ~110 kDa and monomeric structures with a of ~38 kDa. The migration of the heated proteins to locations between 30 and 45 kDa agrees with the monomeric MW. However, the migration of the unheated proteins to positions between 66 and 97 kDa was lower than the estimated trimeric MW. Our later observation suggested that the shift in protein MW was an effect of the detergent present in the Omp samples. It was found that

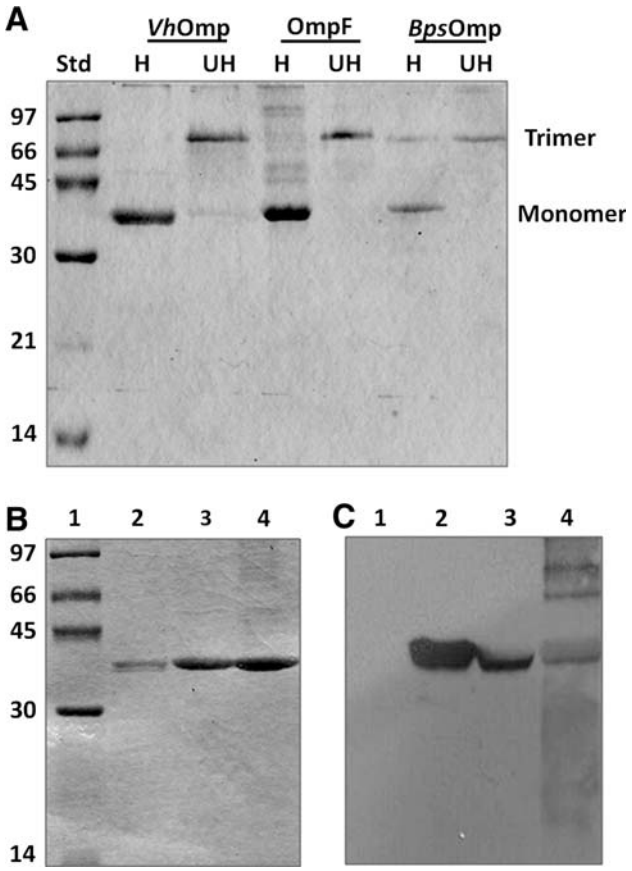

Fig. 1 SDS/PAGE analysis of the isolated outer membrane protein from *V. harveyi*. **a** SDS/PAGE analysis of VhOmp, OmpF, and BpsOmp proteins under heated (H) or unheated (UH) conditions. Samples (3 µg) were prepared in the sample buffer containing SDS for UH proteins or heated at 100°C for 5 min for H proteins. **b** Coomassie stain of the monomeric outer membrane proteins. **c** The corresponding immunoblot of the monomeric proteins using AB_{BpsOmp}. Lane 1, low-MW standard markers; lane 2, VhOmp; lane 3, BpsOmp; lane 4, *E. coli* OmpF

when the MW markers were prepared in 1% (v/v) octyl-POE prior to SDS/PAGE, the Omp bands were seen at the expected position slightly above 97 kDa.

To initially verify that VhOmp is a porin, immunoblot analysis of VhOmp was performed with polyclonal antibodies specific for BpsOmp (AB_{BpsOmp}), a previously identified porin from the tropical pathogen *B. pseudomallei* (Siritapetawee et al. 2004a, b). Immunoblotting (Fig. 1c) showed that VhOmp cross-reacted strongly with AB_{BpsOmp}. Only the VhOmp band was recognized, whereas the MW markers did not react at all with the antibodies. Also, the *Vibrio* protein did not give signal with the rabbit preimmune serum (not shown). Data obtained from SDS-PAGE and immunoblotting suggested that the VhOmp is immunologically, and possibly also structurally, related to the BpsOmp porin. On the other hand, very faint signals detected with OmpF gave an indication that the *E. coli*

Table 1 Mass identification of an outer membrane protein from *V. harveyi* by MALDI/TOF mass spectrometry

Peptide location (start–end)	Observed monoiso-topic mass	Expected monoisotopic mass	Peptide sequence	Identified protein
113–133	2554.02	2554.09	K.HMQDAEMFTNAACMTLNIWDR.F	MOMPF_CHLTR (major outer membrane porin from *Chlamydia trachomati*
134–148	1677.84	1677.81	R.FDVFCTLGATSGYLK.G	
200–222	2543.17	2543.19	R.AALWECGCATLGASFQYAQSKPK.I	
282–292	1281.65	1281.68	R.LNMFTPYIGVK.W	
296–309	1575.80	1575.80	R.ASFDSDTIRIAQPR.L	
357–372	1633.81	1633.83	R.KSCGIAVGTTIVDADK.Y	
358–372	1505.77	1505.74	K.SCGIAVGTTIVDADK.Y	
373–385	1563.80	1563.83	K.YAVTVETRLIDER.A	
381–394	1639.13	1638.89	R.LIDERAAHVNAQFR.F	

Note: Isolated VhOmp was run on 12% SDS/PAGE, then tryptic peptides were prepared by in-gel digestion. Extracted peptides were identified by mass analysis using MALDI/TOF mass spectrometry (BioServiceUnit, Bangkok, Thailand). For protein identification, monoisotopic masses of the peptides were subjected to MASCOT (http://www.matrixscience.com/) for SwissProt database search

porin was immunologically inactive against the BpsOmp antibodies.

Peptide Mass Analysis by Mass Spectrometry

Tryptic peptides of the isolated VhOmp were analyzed by means of MS. Forty-one possible isotopic masses were obtained from MALDI/TOF MS and were then subjected to a MASCOT search for peptide mass identification. The Swiss-Prot database search results displayed several proteins of various sizes that matched the submitted peptides. Nevertheless, nine peptides gave unambiguous mass identity, within 0.01% accuracy, to the corresponding peptides of a major outer membrane porin from *Chlamydia trachomatis* (accession number MOMPF_CHLTR) (Table 1). Since MOMPF from *C. trachomatis* was previously reported to be a functional porin (Findlay et al. 2005), the matched peptides obtained from MALDI/TOF mass identification further confirmed that the isolated VhOmp could potentially be a pore-forming outer membrane protein.

Interaction of VhOmp with Azolectin Bilayers and Pore Conductance Analysis

Azolectin membrane conductance measurements in the mode of the BLM assay were carried out to inspect the interaction of the purified Omp from *V. harveyi* with artificial lipid bilayers and to confirm the pore-forming ability of the likely porin candidate. In a typical test, 1 μg/ml VhOmp was added to the electrolyte on the cis side of a fresh and stable azolectin bilayer that was voltage-clamped to membrane potentials, V_m, of −50 mV at the same time that the membrane current, I_m, was monitored over time. Though not instantaneously, the presence of VhOmp in the lipid bilayer bathing solution routinely triggered step-like increases in I_m, an effect that signified the occurrence of quantal changes in azolectin membrane resistance due to sequential insertion of individual units of VhOmp pores. Current steps were predominantly directed toward higher currents, and as a result of the chronological appearance of a number of events, the signal gradually went beyond the 1000-pA upper limit of the amplifier's headstage, leading to saturation of the current recording and hence termination of the experiment. As expected from the well-known channel-forming character of the *E. coli* OmpF membrane protein, current steps developed in comparable fashion when, in control experiments, OmpF replaced the VhOmp as the active protein component in the added formulation (not shown). Insertions and the related current steps were, however, not observed when buffer solutions without an Omp were added to the cis electrolyte. The conclusion was thus that the mediators of the observed membrane activity in terms of current flow were the macromolecular VhOmp and OmpF units and not other components in their buffer solution.

Figure 2a is an illustration of a representative BLM single-channel recording that was acquired while subjecting an azolectin bilayer, in 0.2 M KCl and at a low membrane potential of −50 mV, to solubilized VhOmp. Successive distinct downward jumps in membrane current are an evident characteristic feature. The current increments displayed, in real time, the consecutive insertions of conductive ion channel-like VhOmp units into the otherwise insulating double-layered lipid partition. This was very consistent with observations made previously under analogous conditions for OmpF (Baslé et al. 2004) and other Omps (Schiffler et al. 2007; Ruíz et al. 2004; Harwardt et al. 2004; Marques et al. 2004; Rieß et al. 2003; Andersen et al. 2003; Ahting et al. 2001; Maier et al. 2001; Dè et al. 2000). A statistical analysis was performed with

data that came from the reaction of many membranes with VhOmp. Using division by the applied membrane voltage, the pooled amplitudes of the recognized current steps were computed as values of step (pore) conductance, which were then collated into conductance histograms using linear scaling with a bin width of 0.2 nS. Figure 2b displays the distribution in the pore conductance of 436 VhOmp insertions in 22 azolectin membranes. Pore populations of various sizes presented reasonably well a single Gaussian distribution with a peak maximum at about 0.9 nS, a value symbolizing the conductance of fully open individual trimeric units of VhOmp. A value of ~1 nS was previously reported as the conductance of fully open trimeric units of the OmpU and OmpT porins from *Vibrio cholerae*, however, the value was assessed in 0.15 M KCl (Duret et al. 2007). The value of 0.9 nS in 0.2 M KCl for VhOmp, but 1 nS in 0.15 M KCl for OmpU and OmpT, is a sign that *V. cholerae* porins may be somewhat larger than their *V. harveyi* analogue. As already mentioned, the conductance of trimeric OmpF channels has been assessed from I/t curves as for VhOmp. Actually, the current increments that came from OmpF membrane insertions were reproducibly larger than those for VhOmp, and statistical analysis of about 170 OmpF current steps in 18 membranes revealed an average conductance of 1.9 nS for individual OmpF insertions (not shown). Under the given experimental conditions, VhOmp channels (0.9 nS) thus appeared not to be as good ion conductors as their OmpF analogues (1.9 nS). Of note, the trimeric OmpF conductance value in this study was higher than those reported in the literature. For instance, An OmpF conductance of ~1 nS was measured in 0.2 M KCl for protein channels that were incorporated in giant liposomes and studied via the patch-clamp technique (Bredin et al. 2002). As the characteristics of the bilayer are known to influence conductance measurements, data from a study by Baslé et al. (2004) may be better for comparison; Basle et al. employed the Montal and Mueller (1972) type of "solvent-free" lipid bilayers and the BLM technique for OmpF channel characterization. While 0.94 nS was reported as the average OmpF trimer conductance in 0.15 M KCl in the study by Basle et al., a value of 1.3 nS has also been observed, for instance, in experiments that dealt with the three-step closing behavior of trimeric protein insertions. The higher trimeric OmpF conductance in our study is associated to some extent with the higher electrolyte concentration (0.2 instead of 0.15 M KCl) but may also be related to differences in the properties of the bilayers, which are solvent-containing, on the one hand, and virtually solvent-free, on the other. Apparently, the experimental conditions used in this study favored the determination of higher OmpF trimer conductance values, but the basic OmpF channel behavior, such as the observable sequential insertion of multiple trimers one after the other and the three-step-like closure of the individual monomers of a trimeric porin unit in response to elevations of membrane potential, were in good accordance with the literature.

Individual VhOmp pores were tested within the range of –100 to +100 mV across the azolectin bilayer for the dependence of their conductance level on the voltage across the hosting lipid bilayer membrane. While the membrane current was monitored, a linear voltage ramp of low scan speed was used after the incorporation of a VhOmp trimer to bring V_m, the membrane potential, slowly from –100 to +100 mV. Figure 3 is a representative plot of the membrane current as a function of the transmembrane voltage, in this case for a VhOmp channel that inserted at –50 mV and induced a current step of 45 pA in 0.2 M KCl. The conductance of the pore (=slope of the *I*/*V* curve) was constant over the entire voltage range scanned, identical for positive and negative potentials on the cis side of the membrane, and 0.95 nS for this particular channel. The average slope conductance derived from voltage ramp experiments was 0.91 ± 0.14 nS ($n = 5$), which is in agreement with the mean value of the channel conductance obtained by analysis of the pooled current increments of VhOmp insertions at 50 mV. The independence of the channel conductance from the applied voltage revealed that VhOmp channels within the limits of ±100mV are not voltage-gated and basically work under this condition as ordinary ohmic electrical conduits. This is comparable to the behavior of the *Vibrio cholera* porins OmpU and OmpT, which also showed, in lipid bilayer experiments, linearity in *I*/*V* curves, but from −170 to +160 mV and from −92 to +92 mV, respectively (Simonet et al. 2003; Duret et al. 2007).

As true for OmpF and many other classic Omps from Gram-negative bacteria, in this study VhOmp appeared as a trimer in biochemical tests under nondenaturing conditions, and it was assumed that it would incorporate in the form of single units of these into lipid bilayers. Biophysical evidence on the trimeric nature of the pore-forming protein was obtained when VhOmp was allowed to incorporate into an azolectin membrane at a low "insertion" transmembrane potential (typically −50 mV) as an open channel, which, after formation, was subjected to an elevated "closing" membrane potential (±150 mV), with the aim of revealing the distinctive sequential closures of the three conducting pores of a trimeric inclusion. Figure 4 illustrates the experimental observation of the predictable three-step closing of reconstituted VhOmp and OmpF trimers. They both converted in three steps from an initially fully open to a fully closed state under the influence of the applied voltage, within the space of a few seconds. It is worth mentioning that the two porins in this study behaved as the *Vibrio cholera* porins OmpU and OmpT, which also

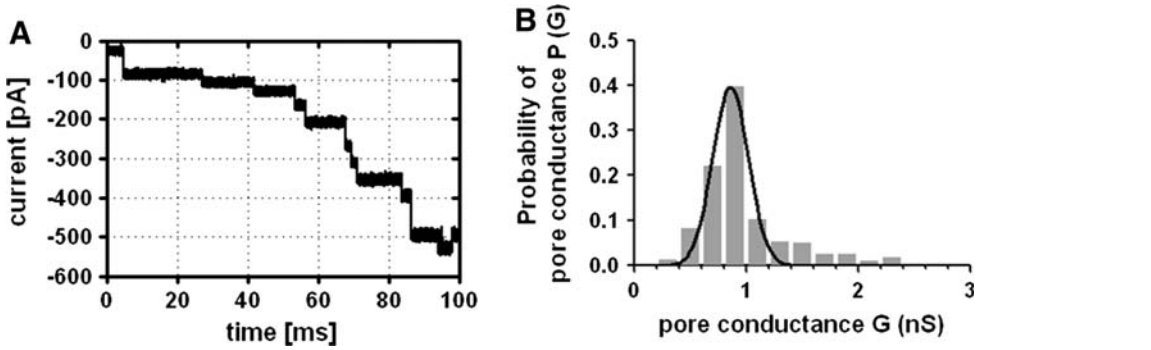

Fig. 2 Measurement of VhOmp pore conductances in 0.2 M KCl. **a** Recording of successive insertion events of pure VhOmp trimers into an azolectin bilayer at a transmembrane potential of –50 mV and a temperature of 25°C. Lipid bilayers were painted with a solution of 50 mg azolectin in 1 ml *n*-hexane and bathed on either side with 0.2 M KCl. VhOmp (1 µg) was added to the 10-ml electrolyte on the cis side to trigger pore insertions. **b** Histograms of the probability of a certain pore conductance (G) derived from VhOmp BLM measurements as shown in A. Results from 436 protein insertions into 22 membranes were pooled and used for the analysis. The probability of pore conductance is defined as the number of events within the given range of G values divided by the total number of events (436). At a probability of 1 thus all G values would be located within one of the specified G ranges. The *black line* refers to a single Gaussian fit

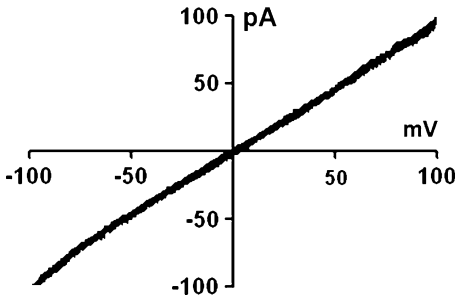

Fig. 3 Current-voltage relationship of VhOmp in symmetrical 0.2 M KCl. The current through a single open VhOmp channel was monitored in 0.2 M KCl while gradually changing the voltage across the azolectin bilayer membrane via a linear voltage ramp from −100 to +100 mV with a scan speed of 5mV/s. From the slope of a linear curve fit (r^2 = 0.998, not shown) the conductance of this particular VhOmp channel was determined as 0.95 nS

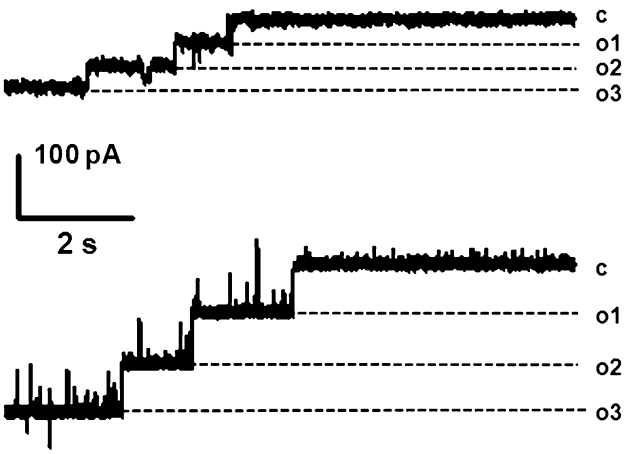

Fig. 4 Successive three-step closures of trimeric pores of VhOmp and the reference porin OmpF. Under the influence of an elevated membrane voltage, V_m, of –150 mV, the initially fully open (*top*) VhOmp and (*bottom*) OmpF trimers changed to the fully closed state. Pore closure was not straight but through a sequential shutdown of the three individual monomers of the structure one at a time. Measurements were achieved at 25°C in symmetric 0.2 M KCl and with 1 µg/ml Omp present on the cis side of a painted azolectin membrane

followed a step-like closing mechanism at elevated membrane potentials, at least in solutions of neutral pH (Duret et al. 2007).

To obtain further insight into the biophysical properties of the VhOmp channel, single-channel BLM experiments were performed, with the bilayer membrane bathed in 1 M KCl, 1 M LiCl, 1 M CsCl, or 1 M Kac, and the results of this set of experiments are summarized in Table 2. In 1 M KCl, the conductance of VhOmp trimers was revealed to be 2.02 nS. Benz et al. (1997) previously reported 2 nS as the conductance for individual monomers of the OmpU trimers from *Vibrio cholerae* classical strain 569B in 1 M KCl, which again suggests that OmpU channels may be larger than their VhOmp analogues. The sequence of the magnitude of the single-channel conductances of the different salts through VhOmp pores was CsCl ≫ KCl ≫ LiCl > Kac. Compared to the value measured in KCl, a change of the mobile potassium to the less mobile lithium cation and of the mobile chloride to the less mobile acetate anion decreased the magnitude of the single-channel conductance of VhOmp insertions to 72 and 65%, respectively. With Li^+ and Ac^- both similarly carrying one elementary charge when moving in between a pair of electrodes of opposite polarity and being close in their effective (ionic) radii and the related mobility in aqueous environments, the assessment of conductance values in 1 M KCl, LiCl, and Kac suggested that VhOmp pores are, if at all, only very moderately selective against the charge of the conducting species, and anions and cations can act as pore users with an almost-equivalent power. Furthermore, the replacement of $K^+$with Li^+ or Cs^+ demonstrated that the permeability

Table 2 Average conductance (G) of VhOmp pores as a function of different salt solutions

Salt	Concentration (M)	G (nS)
$KOOCCH_3$	1	1.33 (46)
LiCl	1	1.46 (36)
KCl	0.05	0.47 (60)
	0.2	0.88 (436)
	1	2.02 (25)
	2	2.57 (28)
CsCl	1	2.90 (30)

Note: Bilayer membranes were formed at 25°C from solutions with 50 mg azolectin in 1 ml *n*-hexane. The number of single insertion events used to calculate the listed average G value is given in parentheses

of cations through VhOmp channels was well in line with the ionic radii and related mobility of these cations in water. Li^+, as the largest, K^+, as the intermediate, and Cs^+, as the smallest solvated ion in this collection displayed the lowest, middle, and highest single VhOmp channel conductances, respectively. As the pore conductance followed the bulk aqueous conductivity of salts reasonably well, VhOmp pores can be thought of as water-filled tubes supporting ion travel.

At a given constant temperature the conductivity of KCl solutions is known to increase in a straight line with the concentration of the salt (Pratt et al. 2001). Accordingly, the conductance of general protein channels that act in lipid membranes without extra functional features as gateways for traveling ions usually also scales linearly with the strength of the electrolyte in the bilayer cell (Schiffler et al. 2007; Ruíz et al. 2004; Harwardt et al. 2004; Andersen et al. 2003; Maier et al. 2001). Table 2 provides a list of the single-channel conductance values of VhOmp pores as a function of the (symmetrical) KCl concentration in the aqueous phase in the two compartments of the bilayer cell. BLM measurements were done in 0.05, 0.2, 1, and 2 M KCl. As evident from the plots in Fig. 5a, b, the single-channel conductance of VhOmp protein channels was not directly proportional to the concentration of the membrane-bathing salt solution but, in contrast, was linearly correlated with the square root of the KCl concentration. A nonlinear relationship between channel conductance and salt concentration has previously been observed for some other Omps from Gram-positive and Gram-negative bacteria (Stegmeier and Andersen 2006; Hünten et al. 2005; Bornet et al. 2004; Rieß et al. 2003), and it was proposed that charged entities at the inner wall of porin channels may be responsible for the deviation from linearity (Hünten et al. 2005; Schirmer and Phale 1999; Lichtinger et al. 1999; Trias and Benz 1994). The actual influence of the ionization state of channel residues on ion permeation has been well demonstrated in a study on the pH dependence of OmpF conductance (Nestorovicha et al. 2003), the role of charged residues in the OmpF porin channel constriction in terms of ion flux has been proved (Phale et al. 2001), and the phenomenon has been subjected to theoretical and computational studies (Roux et al. 2004). Based on our data, a VhOmp pore is likely to have charged internal entities that interact with ionic species and affect their permeation; however, they have not yet been identified.

An interesting observation was made when azolectin membranes were exposed to electrolytes that contained not only VhOmp as an insertable pore-forming polypeptide at levels of 0.1 to 1000 ng/ml but also 200 µg of the polyclonal antibody AB_{BpsOmp} against a porin from the tropical pathogen *B. pseudomallei* (Siritapetawee et al. 2004a, b). Note that the current traces in the presence of the antibodies (Fig. 6b) displayed a lot of transitory closures and, as a result, appeared more "flickery" than those with no antibodies (Fig. 6a). Furthermore, with the recording in the solution with the highest ratio of VhOmp/AB_{BpsOmp} being the only exception, the typical step-like breakdown of membrane resistance induced by VhOmp insertions was completely abolished when the membrane-bathing solution contained supplementary antibodies. Control experiments confirmed the power of even lone 0.1 ng VhOmp in 1 ml *cis* electrolyte to trigger the onset of the distinct quantal current increments associated with active protein channel implementation into the lipid bilayer (Fig. 6a). Wyllie et al. (1998, 1999) previously reported that the presence of oligomer-specific antibodies significantly modulated the channel activity of recombinant major outer membrane porins from *C. psittaci* and *C. pneumoniae*. Reduced pore conductance levels as well as short-lived channel closures were reported as visible effects. Here, AB_{BpsOmp} had a comparable impact on the behavior of VhOmp. The abolishment of step-like changes in membrane current either could come from a solution reaction of the antibodies with pore-forming protein units, and the failure of resulting protein/antibody agglomerates to insert properly into the lipid bilayer membrane, or could originate from an interaction of already inserted VhOmp with the antagonist and an antibody-induced obstruction of ion flow through the conduit. Further studies are needed to clarify this phenomenon.

Biological Activity of VhOmp on Human Red Blood Cells

Knowledge of the capacity of the VhOmp porin to induce perforation of the membrane of living cells and thus cell lysis is helpful for understanding the pathogenicity of *V. harveyi*. A cell lysis assay was therefore carried out to verify the biological activity of VhOmp in vivo. In the experiments, human RBCs were incubated for 5 min at

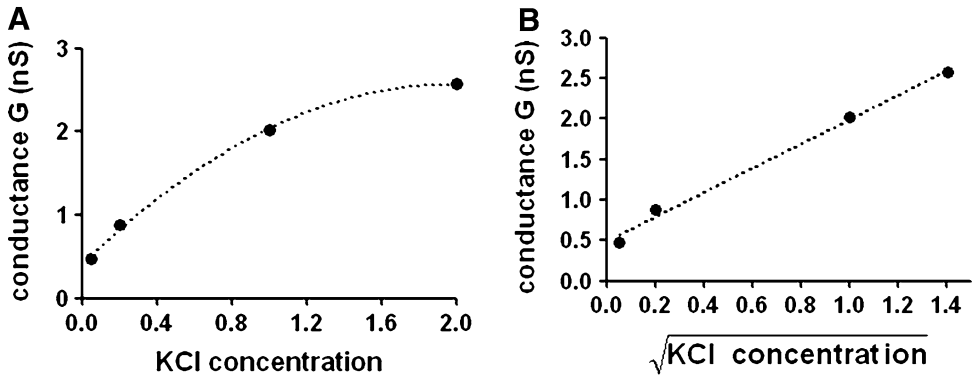

Fig. 5 Dependence of VhOmp single-channel conductance on the concentration of the membrane-bathing electrolyte in the bilayer chambers. The average single-channel conductance of VhOmp was determined for four symmetrical KCl concentrations of 0.05, 0.2, 1, and 2 M (see Table 2). **a** Plot of measured pore conductance, G, versus KCl concentration. **b** Plot of the pore conductance, G, versus the square root of the KCl concentration. For all trials lipid bilayers were painted with a solution of 50 mg azolectin in 1 ml *n*-hexane and symmetrically bathed in the electrolyte of chosen molar concentration. Recording temperature was 25°C, and transmembrane voltage –50 mV

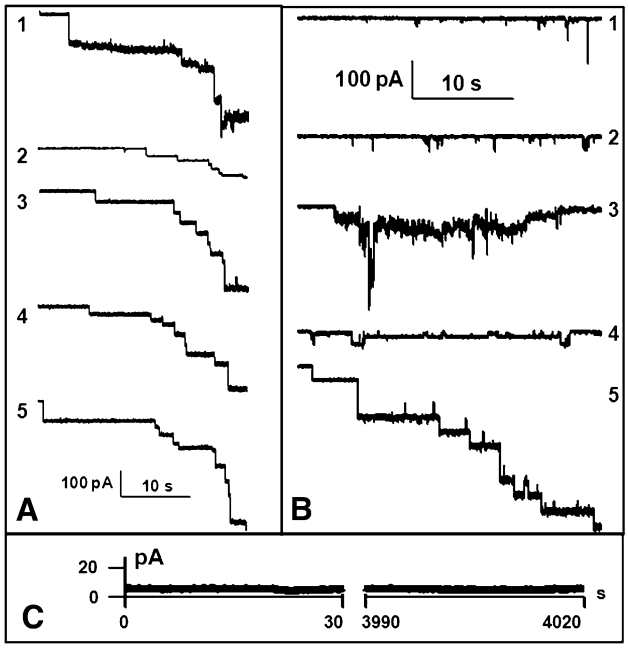

Fig. 6 The effect of outer membrane protein-specific polyclonal antibodies against BpsOmp from the tropical pathogen *B. pseudomallei* on the incorporation of VhOmp channels into azolectin bilayer membranes. **a** Control experiment I: 0.1, 1, 10, 100, and 1000 ng of VhOmp were added to the 1-ml electrolyte on the cis side of the azolectin membrane and led to current traces 1, 2, 3, 4, and 5, respectively. **b** Experiments with AB_{BpsOmp}: 0.1, 1, 10, 100, and 1000 ng of VhOmp plus an extra 200 µg of AB_{BpsOmp} were added to the cis side of the azolectin membrane and led to current traces 1, 2, 3, 4, and 5, respectively. **c** Control experiment II: An azolectin bilayer was formed as usual and then exposed in the absence of VhOmp to 200 µg of AB_{BpsOmp}. For all trials lipid bilayers were painted with a solution of 50 mg azolectin in 1 ml *n*-hexane and symmetrically bathed in 0.2 M KCl. Recording temperature was 25°C, and transmembrane voltage –50 mV

25°C with purified VhOmp or *E. coli* OmpF at various concentrations. Figure 7 shows that the addition of VhOmp caused RBCs to lyse even at a concentration of the protein as low as 0.8 µg/ml. RBCs were completely lysed at VhOmp concentrations of 3.1 and 6.3 µg/ml, indicating strong interactions between the cells (most likely cellular membranes) and the protein. The observation of effective induction of RBC cell lysis by VhOmp is comparable to the demonstration of hemolytic activity of other porins (Calderon et al. 1984). For unknown reasons, the percentage of lysis gradually decreased when RBCs were incubated with higher concentrations of VhOmp proteins (12.5, 25, and 50 µg/ml). Figure 7 also clearly demonstrates that OmpF was much less active than VhOmp since lysis of RBCs was not detected at all at concentrations of the protein <50 µg/ml. Octyl-POE, which was a component in the VhOmp and OmpF cocktails, had no effect on cell lysis. The hemolytic effect of VhOmp on human RBCs is an interesting phenomenon, since *V. harveyi* is known to be a pathogenic bacterium that causes fatal vibriosis in marine animals. Active determinants on the bacterial cell surface, including the outer membrane VhOmp, may be crucial during effective invasion of the bacterium into animal host cells.

In conclusion, an outer membrane protein, namely, VhOmp, was isolated from the marine pathogen *V. harveyi* and functionally characterized as a porin. This trimeric protein inserted well into artificial lipid bilayer membranes and formed ion channels. At low transmembrane potentials VhOmp pores behaved as ohmic conduits. VhOmp displayed biological activity by causing hemolysis of human RBCs. Since itse bacterial species of origin is not harmful to humans, VhOmp is recommended as a good alternative model porin for general Omp structure/function studies. Also, the outcome of this study is the basis for further work focusing on genetically engineered VhOmp variants and combined structural (X-ray crystallography) and functional (BLM) studies. These will be supportive for evaluation of,

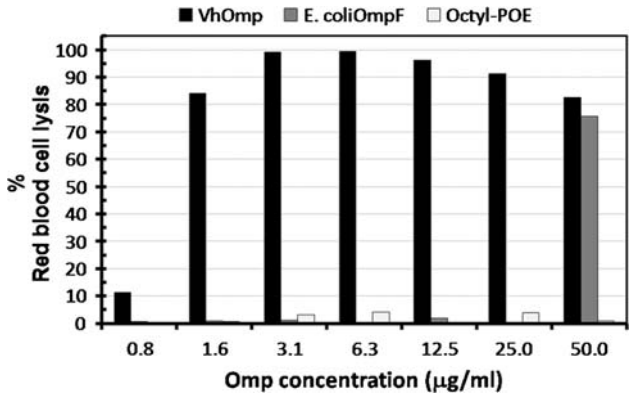

Fig. 7 Effect of VhOmp and OmpF on human red blood cells (RBCs). Three percent human RBCs was added to either VhOmp or OmpF at varied concentrations of 0.8–50 mg/ml and incubated at 25°C for 5 min. Hemolysis of RBCs was determined by measuring a decrease in turbidity of the cell suspension at an optical density of 600 nm. The data are average values obtained from three independent sets of experiments

for instance, the mechanisms behind the flux of antibiotics through Omp pores, in general, and the development of tailored Omp-based vaccines.

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