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Molecular analysis of antimicrobial agent translocation through the membrane porin *Bps*Omp38 from an ultraresistant *Burkholderia pseudomallei* strain

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ABSTRACT

Burkholderia pseudomallei (*Bps*) is a Gram-negative bacterium that causes melioidosis, an infectious disease of animals and humans common in northern and north-eastern parts of Thailand. Successful treatment of melioidosis is difficult due to intrinsic resistance of *Bps* to various antibacterial agents. It has been suggested that the antimicrobial resistance of this organism may result from poor permeability of the active compounds through porin channels located in the outer membrane (OM) of the bacterium. In previous work, a 38-kDa protein, named "*Bps*Omp38", was isolated from the OM of *Bps*. A topology prediction and liposome-swelling assay suggested that *Bps*Omp38 comprises a β-barrel structure and acts as a general diffusion porin. The present study employed black lipid membrane (BLM) reconstitution to demonstrate the single-channel conductance of the trimeric *Bps*Omp38 to be 2.7 ± 0.3 nS in 1 M KCl. High-time resolution BLM measurements displayed ion current blockages of seven antimicrobial agents in a concentration-dependent manner with the translocation on-rate (k_{on}) following the order: norfloxacin ≫ ertapenem > ceftazidime > cefepime > imipenem > meropenem > penicillin G. The dwell time of a selected antimicrobial agent (ertapenem) decayed exponentially with increasing temperature. The energy barrier for the ertapenem binding to the affinity site inside the *Bps*Omp38 channel was estimated from the Arrhenius plot to be 12 kT and for the ertapenem release to be 13 kT at + 100 mV. The BLM data obtained from this study provide the first insight into antimicrobial agent translocation through the *Bps*Omp38 channel.

1. Introduction

Burkholderia pseudomallei (*Bps*) is a soil-dwelling Gram-negative bacterium commonly found in Southeast Asia and Northern Australia and a cause of a deadly disease of mammalian species termed melioidosis [1–4]. Patients infected with *Bps* usually develop skin ulcers, visceral abscesses, pneumonia and septicemia that imperatively require immediate antimicrobial treatment to avoid fatal progression of the disease. Very often, antimicrobial treatment is quite a challenge due to the high intrinsic broad spectrum resistance that most *Bps* strains exhibit towards a broad spectrum of antimicrobial agents including but not limited to β-lactam antibiotics, aminoglycosides, macrolides, and cephalosporins [1–4]. Due to the high incidence of drug resistance and very high virulence, *Bps* is regarded a potential bioterrorism and warfare agent [5]. As such, this organism has been listed by the US Centre for Disease Control and Prevention as a category B health hazard [6,7]. Clinical and security concerns associated with *Bps* have justified intensive research in attempt to address the structural and functional organization of this pathogen as a prelude to the design of novel and efficacious anti-*Bps* therapeutic agents. Most of ongoing studies include characterization of biological and pathophysiological aspects of the agent, unraveling the mechanisms of genomic plasticity and evolution, as well as understanding the molecular mechanisms underlying drug resistance. The publication of the sequences of the entire *Bps* genome [8], as well as data from drug susceptibility testing [9–12], and recent reports on *Bps* vaccines [13–15] are important developments in the search for an effective anti-melioidosis treatment.

Current scientific evidence suggests that *Bps* successfully utilizes the strategy of genetic evolution, modified protein expression and/or mutation more than most other bacteria. These features underscore the ability of the pathogen to establish troublesome resistance against many antimicrobial agents. Some of the mechanisms of drug resistance include: alteration of intracellular drug action sites, generation of enzymes for the modification and/or complete degradation of drug molecules within the cytosol, and restriction of intracellular drug accumulation through impaired uptake and/or enhanced drug efflux [16–19].

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In the present study, we addressed limitation of cellular drug uptake which in a number of other bacteria is associated with reduced molecular transport of the active compounds through bacterial outer membrane protein (Omp) channels. Also known as porins, Omp channels are typical β-barrel protein structures that independently or as oligomeric units are inserted into the outer lipid bilayer of the bacterial cell wall to form pores through which extracellular species can diffuse and gain access to the periplasmic space and cytosol [20–22]. Membrane trafficking via porins has been elegantly measured at molecular level using the black lipid membrane (BLM) technique [23–25]. In BLM experiments, the parameter measured is the voltage-induced charge flow through an artificial lipid bilayer membrane that separates two electrolyte compartments and one that has the study porin artificially inserted. Recordings of the transmembrane current at microsecond time resolution enable visualization of insertions of the porin in their open state as stepwise increases in the signal. Movement of individual drug/other molecules through a stably open unit is detected by means of transient current fluctuations derived from physical channel blockade during the residence time of compounds traveling through the porin. Mechanistically, bacterial cells can counteract the influx of drug molecules through reduction of the total number of porins in their outer membrane, a decrease in the cross-sectional dimension of the protein channels, and/or through modification of the electrostatics and/or hydrophobicity of the pore interior through point mutations of the amino acids lining the protein pores. Quantitative correlation of the entry of specific drug molecules with a firm structural and predictive computational analysis may lead to a better understanding of the molecular basis of antimicrobial agent permeability, facilitating the design of effective drugs that have greater penetrating power [26].

While functional [27–32] and computational [33–35] BLM studies focused on membrane drug permeation have been performed on a number of bacterial porins, no similar research has investigated analogous proteins from clinically important *Bps* strains. In previous work, an Omp with an apparent molecular weight (MW) of 38 kDa, referred to as *Bps*Omp38, was isolated from the *Bps* cell wall [36,37]. Topology prediction and molecular modeling suggested that *Bps*Omp38 has a β-barrel structure, a feature that is common among membrane porins. Subsequently, a liposome-swelling assay on this protein confirmed its channel-forming properties [37]. In the present study, a detailed functional characterization of the *Bps*Omp38 porin by BLM-based single ion-channel analysis is described. Ion current measurements were carried out in the absence and in the presence of seven different antimicrobial agents as additional diffusing species in the membrane-bathing electrolyte. The differences in the ability of the selected drugs to move through open *Bps*Omp38 pores and their kinetic behavior are discussed in the context of molecular drug/porin interactions.

2. Materials and methods

2.1. BpsOmp38 expression and purification

The *E. coli* strain BL21 (DE3) Omp8 was a gift from Ralf Koebnik, Laboratoire Génome et Développement des Plantes, Universit é de Perpignan via Domitia, Montpellier, France. This strain of *E. coli* does not express the major outer membrane proteins LamB, OmpA, OmpC and OmpF [38]; it was experimentally transformed with the recombinant plasmid pET23d.*Bps*Omp38. Purification of the recombinant *Bps*Omp38 followed a modified version of protocols described by Garavito and Rosenbusch [39] and Rosenbusch [40]. In brief, transformed cells were grown at 37 °C in Luria–Bertani (LB) liquid medium containing 100 μg/ml ampicillin. At an OD_{600} reading of 0.5, IPTG (isopropyl β-D-thiogalactoside) was added to a final concentration of 0.4 mM. Cell growth was continued for a further 6 h and then cells were harvested by centrifugation at 2948 × *g* for 10 min. The cell pellet was resuspended in buffer containing 20 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μg/ml DNase I and 10 μg/ml RNase A and then disrupted using a high-pressure homogenizer (EmulsiFlex-C3, Avestin Europe, Mannheim, Germany). The recombinant *Bps*Omp38 was further extracted from the peptidoglycan layer using sodium dodecyl sulfate (SDS)-containing solutions based on a procedure reported by Lugtenberg and Alphen [41]. Briefly, SDS was added to the cell suspension to a final concentration of 2% (v/v) and incubation was carried out for 1 h at 60 °C with gentle shaking. The crude extract was then centrifuged at 39,636 × *g* for 60 min at 4 °C. The pellet, which at this stage included the cell envelopes, was resuspended in 20 mM phosphate buffer, pH 7.4 (PBS), containing 0.125% (v/v) octyl-POE (*n*-octyl-polyoxyethylene; ALEXIS Biochemicals, Lausanne, Switzerland). The suspension was incubated at 37 °C with gentle shaking for 60 min and then centrifuged at 109,564 × *g* at 4 °C for 40 min. The new pellet, now rich in outer membranes, was resuspended in 20 mM phosphate buffer, pH 7.4 containing 3% (v/v) octyl-POE and the suspension incubated at 37 °C with shaking at 250 rpm for 1 h to solubilize the porin. Insoluble material was removed by centrifugation at 109,564 × *g* at 20 °C for 40 min and the porin-rich supernatant concentrated using Amicon Ultra-15 centrifugal filter devices with a nominal MW limit of 30 kDa (Millipore, Schwalbach, Germany). Amicon centrifugal filters were also used to exchange the original preparation buffer with 20 mM PBS, containing 1% (v/v) octyl-POE. Aliquots of the final protein sample were used for absorbance measurement at 280 nm for the determination of protein concentration using NanoDropT 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) for the assessment of sample purity.

2.2. Lipid bilayer measurements and single-channel analysis

The following chemicals were used: NaCl, KCl, MES, *n*-pentane, and hexadecane (Sigma-Aldrich, Hamburg, Germany); ceftazidime, norfloxacin, and penicillin G (Sigma-Aldrich); cefepime, imipenem, meropenem, and ertapenem (Basilea Pharmaceutica Ltd., Basel, Switzerland); and 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL, USA). Double distilled and deionized water was used to prepare chemical reagents and the freshly made solutions passed through a 0.4-μm filter. The drug stock solutions for translocation experiments were prepared with 1 M KCl in electrolyte buffer (20 mM phosphate buffer, pH 7.0 or in 20 mM HEPES, pH 8.0).

Lipid bilayer measurements and single-channel analysis were performed as described elsewhere [27–32]. Briefly, a cell with a 40–60 μm diameter aperture in a 15-μm-thick Teflon partition provided a two-compartment black lipid membrane (BLM) chamber and two silver–silver chloride electrodes at either side of the dividing wall allowed voltage control of solvent-free planar lipid bilayers that were formed using a solution of DPhPC in pentane. Low levels of the study *Bps*Omp38 channel were introduced to the *cis* or *trans* side of the bilayers by adding the protein stock solution of about 1–2 μg/ml containing 1% (v/v) octyl-POE (ALEXIS, Switzerland). In the trials addressing the temperature dependence of drug translocation, a peltier element (Dagan Corporation, Minneapolis, MN, USA) was used for accurate temperature regulation of the BLM chamber. At an applied transmembrane voltage of +50 mV, spontaneous channel insertion was usually obtained within a few minutes after adding the protein solution. Conductance measurements were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in the voltage clamp mode and the internal filter at 10 kHz. Amplitude, probability, and single-channel analyses were performed using pClamp v.10.0 software (Molecular Devices). Control experiments (refer to Supplementary S3, upper left recording) showed no