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Lipopolysaccharide promoted opening of the porin channel

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We show here that the imipenem (a carbapenem, β-lactam antibiotic)-permeable porin channels (protein D2 or OprD2) of *Pseudomonas aeruginosa* were closed mostly in the lipopolysaccharide (LPS)-free membrane and were openable by adding LPS to the membrane as assayed by ion conductivity measurements using planar lipid bilayers. Open and closed states of the OprD2 channels exhibited conductivities of about 400 and 30 pS, respectively, in 1 M NaCl. The OprD2 channel in the LPS-containing membrane showed very rapid opening and closing events in a second order and the duration of closure became longer at low membrane potentials.

Channel; Gate; Porin; Conductivity; Antibiotic, Lipopolysaccharide

1. INTRODUCTION

Porins are the channel-forming membrane proteins primarily found in the outer membrane of gram-negative bacteria [1,2]. The water-filled porin channel has low stereospecific solute selectivity, but discriminates solutes according to their size [1,2]. Ion conductivity measurements of *Escherichia coli* porins showed that the channels were opened permanently [3], but closed as the transmembrane potential exceeded critical values [4] or when added the membrane-derived oligosaccharide [5]. Patch-clamp studies of large spheroplasts revealed that *E. coli* porins were mostly closed, but openable by applied hydrostatic pressure [6].

Pseudomonas aeruginosa, which is a pathogen to immunocompromised patients, produces three porins, namely proteins C, D2 and E1 (OprC, D2, E1) [7]. These allow the diffusion of saccharides of M_r less than 350–400 [7]. Protein F is reportedly a porin but this is a matter for debate. The broad spectrum of antibiotic resistance of *P. aeruginosa* is probably attributable to its low antibiotic permeability [8]. OprD2 is unique among the porins, since this protein is consistently missing in the mutants resistant to imipenem, suggesting that OprD2 facilitates imipenem diffusion [9,10]. Antibiotic permeability through this porin is extremely poor in vitro, except for imipenem [11]. The channel forming activity of OprD2 became 10-fold higher as it was treated with trypsin suggesting that trypsin cleaved off the gate-forming domain, leaving the channel forming domain intact [12].

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2. MATERIALS AND METHODS

2.1. *Purification of OprD2*

We purified OprD2 by DEAE-chromatography in non-ionic surfactants as described in [7]. OprD2 was kept in 5 mM octaethyleneglycol dodecylether ($C_{12}E_8$), 150 mM NaCl, 10 mM *N*-2-hydroxyethyl piperazine *N'*-2-ethane sulfonic acid (HEPES) buffer, pH 8.0.

2.2. *Formation of planar lipid bilayers and conductivity measurement*

We formed planar lipid bilayers by the procedure described earlier [3]. A Teflon chamber, separating two aqueous phases by a thin Teflon film (25 μm) with an orifice of 0.3–0.4 mm in diameter was used. The orifice edge was precoated with 0.3 μl of 2 mg L-α-diphytanoylphosphatidyl choline (diPhy-PC) per ml of hexane. We took 2 μl of 10 mg diPhy-PC per ml of n-decane on a brass wire sleeved with a Teflon tubing and lightly applied it onto an orifice of the septum immersed in the salt solution in the chambers. Electrical capacitance increased to about 0.5 $\mu F/cm^2$ within a few min, indicating bilayer formation. Electrical resistance of the membrane appeared to be consistently higher than 400 $G\Omega/cm^2$ Protein to be tested was added to the *cis* compartment in which a 10 GΩ probe was immersed. This was stirred by a magnetic bar for 60 s and the conductivity was recorded through Ag-$AgCl_2$ electrodes connected to a patch-clamp amplifier CEZ-2200 (Nihon-Kohden) by the voltage-clamping mode. Membrane potential was applied through a stimulator SEN-3301 (Nihon-Kohden). Signals were recorded by a digital recording oscilloscope 2232 (Tektronix) and a pen recorder LR4201 (Yokogawa).

2.3. *Preparation of lipopolysaccharide (LPS)*

We purified the LPS from *Salmonella typhimurium* TA2168 producing a deep rough LPS by the procedure described in [13]. An aqueous suspension of LPS was subjected to extensive electrodialysis until the current stabilized at less than 1 mA at 1,000 V. It was then lyophilized, dissolved in n-decane and mixed with diPhy-PC. This was used to form lipid bilayers as above.

3. RESULTS AND DISCUSSION

To provide direct evidence that OprD2 is the channel-forming protein, we added 4×10^{-12} mol of highly purified OprD2 (Fig. 1) into a *cis* compartment of the chambers divided by a planar lipid bilayer. We observed

small stepwise conductance increments in the membrane containing diPhy-PC (Fig. 2a). The conductance ranged from 20 pS to about 100 pS in 1 M NaCl. The most frequent channel conductance appeared to be 20 to 30 pS (Fig. 2b). We also observed these small channels most in the bilayers formed from asolectin (data not shown). It seems difficult that these small channels accommodate the diffusion of saccharides and imipenem. Moreover, we consistently observed large channels ranging from about 200 to 500 pS in 1 M NaCl in the bilayers formed from diPhy-PC (data not shown) at the frequency of about 2–3% of that of the small channel. Our interpretation of these results was that over 95% of the OprD2 channels exist in closed states, and only a small proportion of the channels are open in membranes containing diPhy-PC or asolectin. We reported recently that OprD2 showed a 10 times higher solute permeability when treated with trypsin, suggesting that the gate-forming domain was cleaved off[12]. This and the above results suggest that in the liposome assay, we might be determining the diffusion of sac-

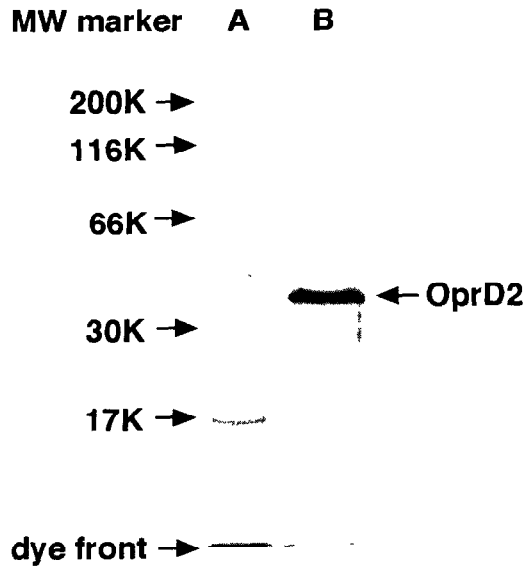
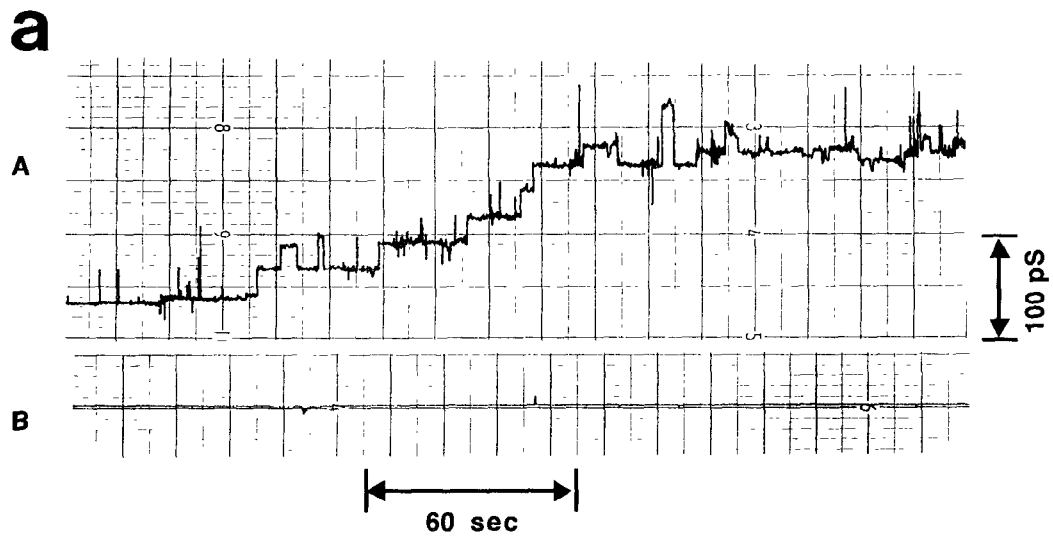


Fig. 1. SDS-acrylamide gel electrophoretogram of highly purified protein D2. Lane A: molecular weight markers. Lane B: 3 μg of purified OprD2.



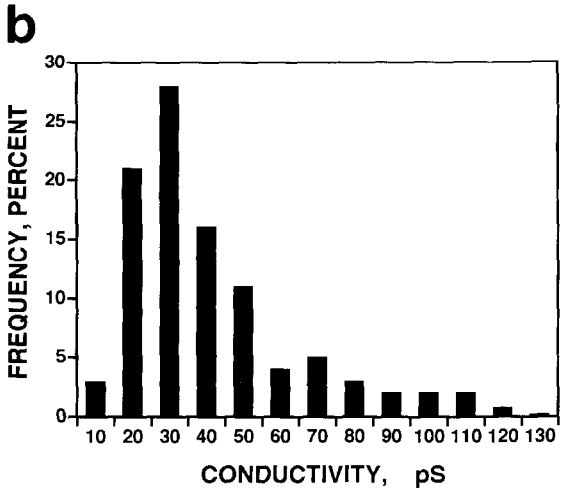


Fig. 2. Channel conductivity of OprD2 in diPhy-PC membrane. (**a**) Typical conductivity increments by OprD2 in the LPS-free membrane A: OprD2 (4×10^{-12} mol /ml) was added. The bath solution contained 1 M NaCl-2 5 mM 2(*N*-morpholino)ethane sulfonic acid (MES)-NaOH buffer, pH 5.9. An electrical potential of +100 mV was applied. B: conductivity of the lipid bilayer as 15 nmol of $C_{12}E_8$ per ml was added. (**b**) Distribution of OprD2 channel conductance in the LPS-free membrane. Conductance of OprD2 measured in planar membranes containing diPhy-PC only was recorded over 400 channel events

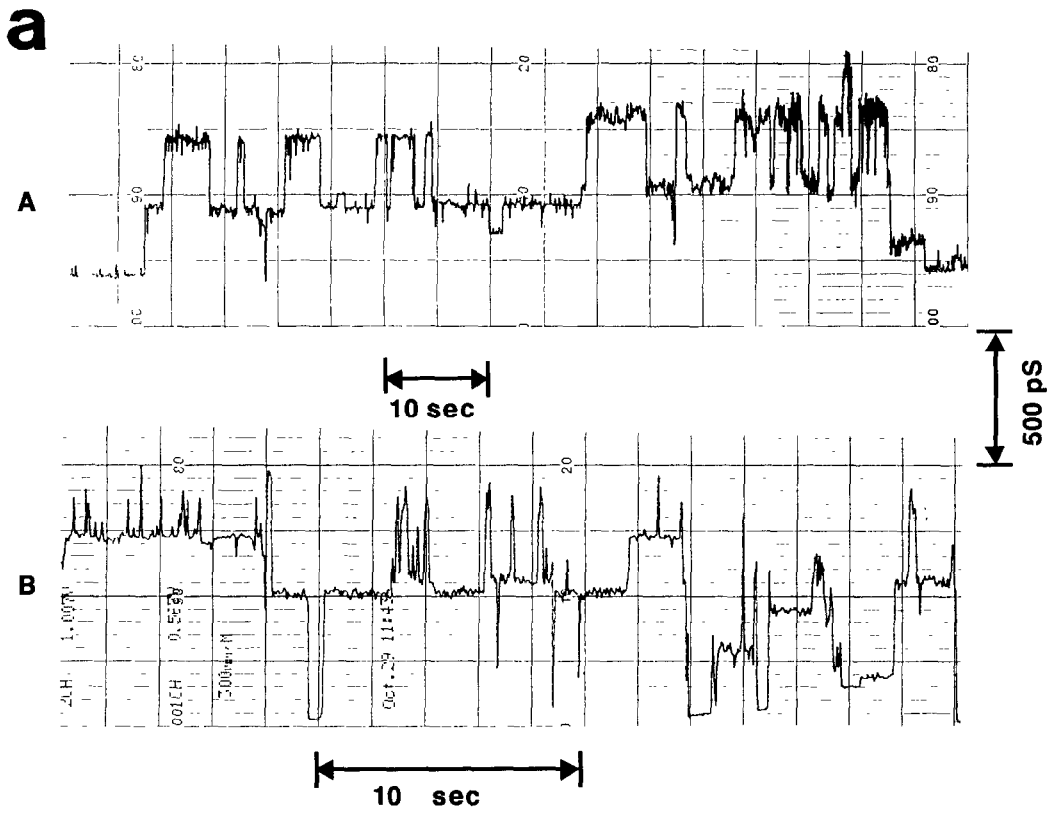
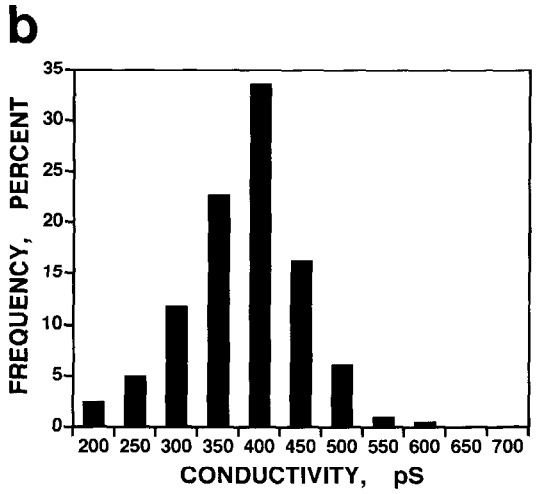


Fig. 3. Conductance of the OprD2 channel in the presence of LPS. (a) Two examples with different chart speeds are shown. Bath solution used was 1 M NaCl, 5 mM $MgCl_2$, 2.5 mM MES-buffer, pH 5.9. The applied membrane potential was +100 mV (b) Histogram of the OprD2 channel in the presence of LPS.



charides and imipenem through only a fraction of the OprD2 channel, namely through those in the open state.

In search of the component(s) that opens the OprD2 channel, we reconstituted planar lipid bilayers from a mixture of LPS and diPhy-PC in n-decane. As we added about 6×10^{-12} mol of OprD2, we observed an abrupt increase in membrane conductance and the membrane broke soon after. Reducing the amount of OprD2 to the small level of 1.5 to 3×10^{-13} mol, we observed discrete channel activity (Fig. 3a). The amount of protein required was about 1/20 of that used in the LPS-free membrane. Under these conditions, we predominantly observed large channels. A histogram showed that the most frequent channel appeared to be 350–400 pS (Fig. 3b). This value is higher than that of the channel conductance of another *P. aeruginosa* porin, OprE1 [14], and lower than that of the *E. coli* porins [3,4]. The conductance appearing in the LPS-containing membrane was more than 10 times larger than that in the LPS-free membrane. A low frequency of appearance of large channels in the diPhy-PC or asolectin membrane might have been attributable to a small amount of LPS contaminating in the porin preparation. The OprD2 channels reconstituted in the LPS-containing planar membranes showed the highest frequency of opening and closing in a second order (Fig. 4a), ever documented among the porin channels except for one very recent report [15]. We consistently recorded this high gating activity in the channels in the presence of LPS suggesting that LPS promotes the gating activity of the

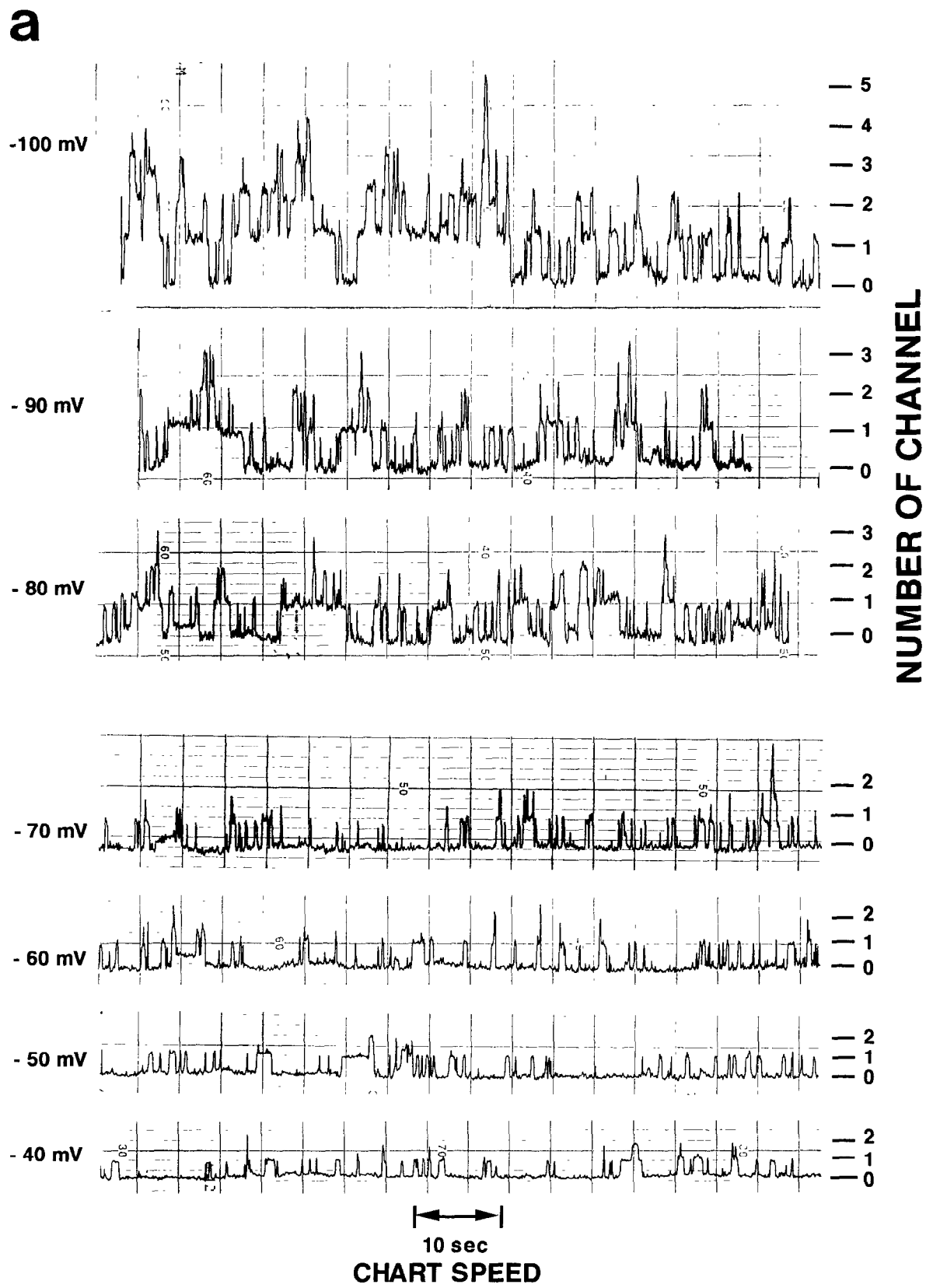


Fig. 4. Potential-dependent opening and closing of the OprD2 channel in the LPS-containing membrane. (a) Closing of the open state of the channel by lowering the membrane potential When several channels were incorporated, negative potential was lowered from −100 mV at 10-mV intervals in a stepwise manner. The figure shows the channel activity with only the relevant charts given for simplicity. We recorded the conductances without adjusting the recorder sensitivity as the membrane potential was changed and therefore marked only the number of channels opened.

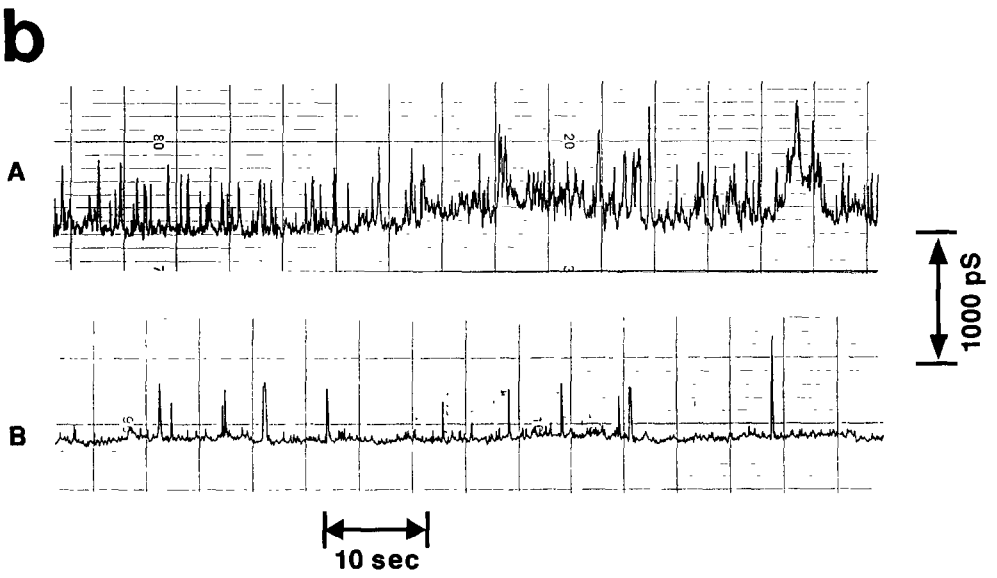


Fig. 4. (continued). (**b**) Voltage-dependent opening of the OprD2 channel. After incorporation of the channels at +100 mV, the membrane potential was lowered to zero and the negative potential was increased stepwise at 10-mV intervals. The figure shows only −100 mV (A) and −50 mV (B) for simplicity. Bath solution contained 1 M NaCl, 5 mM $MgCl_2$, 2.5 mM MES, pH 5.9.

OprD2 channel. We observed that the probability of opening of this channel became much lower when the negative membrane potential became lower than 50 to 70 mV (Fig. 4a). The channels were openable by raising the membrane potential over about −60 mV (Fig. 4b). The effect of positive membrane potential on opening and closing of the OprD2 channel appeared to be less significant as reported for another porin [15]. The gating activity of the OprD2 channel is consistent with our recent findings that OprD2 is fabricated with the gate forming domain whose removal by trypsin treatment resulted in the channel being activated [12].

OprD2 is thought to be responsible for the selective passage of imipenem, and basic amino acids [16]. We have attempted to test the effect of imipenem, L-lysine, L-arginine and L-histidine on OprD2 channel activity. We were unable to detect a change in channel activity in the presence of 10 mM imipenem or 100 mM or less of the above-mentioned amino acids.

Thus, it is highly possible that LPS and possibly other cellular component(s) regulate the opening and closing of the OprD2 gate controlling the solute passage through the channels in the outer membrane. To the best of our knowledge, the data presented in this paper have demonstrated for the first time that LPS promotes opening of the gate for the porin channel, and that low membrane potential tends to close the open state of the channels. High frequency of opening and closing of the channel is unusual among porins.

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