The *Enterobacter aerogenes* outer membrane efflux proteins TolC and EefC have different channel properties

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Abstract

The outer membrane proteins TolC and EefC from *Enterobacter aerogenes* are involved in multidrug resistance as part of two resistance-nodulation-division efflux systems. To gain more understanding in the molecular mechanism underlying drug efflux, we have undertaken an electrophysiological characterization of the channel properties of these two proteins. TolC and EefC were purified in their native trimeric form and then reconstituted in proteoliposomes for patch-clamp experiments and in planar lipid bilayers. Both proteins generated a small single channel conductance of about 80 pS in 0.5 M KCl, indicating a common gated structure. The resultant pores were stable, and no voltage-dependent openings or closures were observed. EefC has a low ionic selectivity ($P_K/P_{Cl} = \sim 3$), whereas TolC is more selective to cations ($P_K/P_{Cl} = \sim 30$). This may provide a possible explanation for the difference in drug selectivity between the AcrAB-TolC and EefABC efflux systems observed *in vivo*. The pore-forming activity of both TolC and EefC was severely inhibited by divalent cations entering from the extracellular side. Another characteristic of the TolC and EefC channels was the systematic closure induced by acidic pH. These results are discussed in respect to the physiological functions and structural models of TolC and EefC.
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Keywords: E. aerogenes; Outer membrane; Multidrug resistance; Efflux pumps; Channel-forming proteins

1. Introduction

Gram-negative bacteria have evolved several specialized pathways for protein export and drug efflux to the extracellular environment. All the pathways require one or more outer membrane proteins, which, in the case of type I secretion systems and resistance-nodulation-division (RND) efflux pumps, belong to the TolC family [1]. Among RND pumps, the AcrA-AcrB-TolC from *Escherichia coli* and MexA-MexB-OprM from *Pseudomonas aeruginosa* are the best-studied representatives and are largely responsible for multidrug resistance (MDR) in these species. These systems consist of an inner membrane transporter (AcrB, MexB), a periplasmic linker protein (AcrA, MexA), and an outer membrane channel (TolC, OprM). The three-component complex creates a sealed proteinaceous exit duct across the two membranes and allows the extrusion of substrates straight through the inner membrane to the external medium. Crystal structures of the three components of a multidrug efflux pump are now available [2–6]. These led to major progress in the understanding of the principles underlying pump assembly and operation, and presented pumps as new targets for drug design. TolC assembles into a homotrimer with a unique "channel-tunnel" structure. It is made up of a 40-Å-long β-barrel anchored in the outer membrane (the channel domain), prolonged by a 100-Å-long α-helical barrel projecting into the periplasmic space (the tunnel domain). The TolC homotrimer forms a single pore with an average accessible interior diameter of 19.8 Å through the whole channel and most of the tunnel. However, in the lower half of the tunnel, three of the six pairs of α-helices fold inward to narrow the proximal (periplasmic) entrance to an effective

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diameter of 3.9 Å, which is too small for substrate access. OprM and OprN from *P. aeruginosa* and VceC from *Vibrio cholerae* are highly homologous to *E. coli* TolC and also exist as trimers [3,7,8]. Purified trimeric TolC and OprM display pore-forming activities when inserted in planar lipid bilayers, with an average single-channel conductance of about 80 pS in 1 M KCl [9–11]. This small conductance reflects the closed steady state of the channel. Transition to open state is a key event in the operation of efflux pumps. It has been proposed that TolC opens by an allosteric "iris-like" uncoiling and realignment of the entrance helices during substrate translocation [5,12,13]. TolC's three-dimensional structure showed that its proximal helices are constrained in a resting closed state by a circular network of intra- and inter-protomer salt bridges and hydrogen bonds [5]. Consistent with this notion, substitutions at important residues can widen or lock the TolC's aperture [12–14]. However, some data are less clear since none of these connections is conserved in *V. cholerae* VceC [7].

Enterobacter aerogenes is a commensal Gram-negative bacterium of the human intestinal flora that has emerged as an important nosocomial pathogen [15]. *E. aerogenes* strains isolated from hospitalized patients generally exhibit high levels of resistance to a wide variety of antibiotics, including β-lactams, quinolones, chloramphenicol and tetracyclines [16,17]. To date, we identified two multidrug efflux pumps, i.e. AcrAB-TolC and EefABC, in *E. aerogenes* [18,19]. The *acrAB* and *tolC* genes are constitutively expressed in wild-type strains and their overexpression contributes to MDR (MultiDrug Resistance) in several clinical isolates [18,20,21]. The *eefABC* locus is cryptic under standard laboratory growth conditions. Nevertheless, the production of EefABC from a multicopy plasmid confers substantial restoration of antibiotic resistance to *E. coli* and *E. aerogenes acrAB* or *tolC* mutants [19].

The present work provides a detailed comparative study of channel properties of purified TolC and EefC from *E. aerogenes* using a combination of electrophysiological techniques. The patch-clamp approach allows the detection of single channels and provides a high-resolution analysis of channel behavior. The planar lipid bilayer approach was used for multi-channel experiments, near physiological conditions. This allowed the analysis of a population of channels subjected to different voltage, pH, and ion species.

2. Materials and Methods

2.1. Bacterial strains and plasmids

Bacterial strains were routinely grown at 37 °C in Luria–Bertani medium (LB) or on solid agar obtained by the addition of 1.5% (w/v) Bacto-Agar. When necessary, antibiotics were added in the growth media at the following final concentrations: ampicillin, 200 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml. *E. coli* strains XL1-Blue (Stratagen, La Jolla, CA, USA) and BL21 (DE3)pLysS (Novagen, Madison, WI, USA) were used for gene cloning and expression of genes under T7 promoter control, respectively.

pMM762 is a pET24a$^+$ derivative encoding TolC-His$_6$ [22]. *eefC* was amplified by PCR without its signal sequence and without the N-terminal cysteine residue of the mature sequence from pEP784 [18] as template with primers *Msc*I-*eefC* (5′ TGGCCAGTGTCTTTGACCCGCACT 3′) and *eefC*-*Sal*I (5′ GTCGACACTGGCGCCTCCCAGAGATT 3′). The forward primer

contains an *Msc*I restriction site (underlined), and the reverse primer contains a *Sal*I restriction site (underlined) to clone the amplification product in frame with the *pelB* N-terminal signal sequence and the His$_6$ C-terminal sequence in pET22b$^+$ (Novagen). PCR reaction was 35 cycles of 30 s at 96 °C, 1 min at 60 °C, and 1.5 min at 68 °C. The PCR product was gel-purified and cloned into pGEM-T (Promega, Madison, WI, USA). The *Msc*I–*Sal*I fragment was then inserted into *Msc*I–*Xho*I restricted pET22b$^+$ to obtain pMM33.

2.2. DNA techniques

Plasmid DNA was purified by using a Wizard® Plus SV Minipreps DNA Purification System kit (Promega) for sequencing. DNA fragments were gel-extracted by using a Concert Rapid Extraction kit (Invitrogen, Cergy Pontoise, France). PCRs were carried out with Elongase® polymerase (Invitrogen). DNA fragments were sequenced using a dRho Terminator cycle Sequencing Ready reaction kit and an ABI PRISM sequencer (Perkin Elmer, Norwalk, CT, USA) and a combination of universal and custom-synthesized primers.

2.3. TolC and EefC purification

E. coli strains BL21(DE3)pLysS carrying pMM762 or pMM33 were grown at 37 °C in LB broth to an OD$_{600}$ of 0.6 and induced with 1 mM IPTG for 3 h. Cells were broken by sonication, membranes were collected at 100,000×*g* for 60 min and washed with 20 mM Tris–HCl pH 7.4, 30 mM MgCl$_2$, 0.5% Triton X-100 to eliminate inner membrane fractions. TolC and EefC were extracted from the outer membrane enriched fractions with 20 mM Tris–HCl pH 7.4, 5% Triton X-100 and insoluble material was removed at 50,000×*g* for 20 min. TolC and EefC were then purified from the supernatant by binding onto a 5-ml HiTrap Chelating column (Amersham Biosciences) charged with Ni^{2+} and eluted with increasing concentrations of imidazole in 20 mM Tris–HCl pH 7.4, 0.5% Triton X-100 [22]. Protein concentrations were determined by using the RC DC protein assay (Bio Rad, Hercules, CA, USA) according to the manufacturer's instructions. The purity was assessed by silver staining after SDS-PAGE.

2.4. Reconstitution of TolC and EefC in planar lipid bilayers

From a 0.5% solution of azolectin (type IV-S, Sigma) in hexane, virtually solvent-free planar lipid bilayers were formed by the apposition of two monolayers on a 125-μm diameter hole in a thin Teflon film (10 μm) sandwiched between two half-glass cells and pretreated with hexadecane/hexane (1:40, v/v). Voltage was applied through an Ag/AgCl electrode in the *cis* side with the *trans* side grounded. The electrolyte solution was 10 mM HEPES pH 7.4, 0.5 M KCl. TolC and EefC were diluted in 0.5% Octyl-POE and then added to the *cis* compartment at the final concentration of about 5 ng/ml. All experiments were performed at room temperature.

In voltage-gating experiments, membranes were subjected to slow ramps of potential (10 mV/s) and transmembrane currents were fed into an amplifier (BBA-01; Eastern Scientific, Rockville, Md.). Current–voltage curves were stored on a computer and analyzed with Scope software (Bio-Logic, Claix, France).

For ion selectivity measurements, zero-current potentials were determined by establishing a 10-fold KCl gradient (1 M:100 mM *cis/trans*) across the bilayer. The ion selectivity was characterized by the ratio P_{cation}/P_{anion} (i.e., the ratio of the permeability for cations to the permeability for anions), calculated using to the Goldman–Hodgkin–Katz equation.

2.5. Reconstitution of TolC and EefC into liposomes and patch-clamp recordings

Purified protein in Triton X-100 was incubated with small unilamellar vesicles of azolectin (Sigma) at a protein to lipid ratio of 1:2,000 (w/w) for 30 min at room temperature before the addition of Bio-Beads SM2 (Bio-Rad, Ivry sur Seine, France) (200 mg/ml) to get rid of detergent. After 3 h of incubation at room temperature, the suspension was centrifuged for 40 min at 33,700×*g*. The pellet was resuspended in 20 μl of 10 mM HEPES pH 7.4, 0.5 M KCl and aliquots were subjected to dehydration/rehydratation procedure to obtain giant proteoliposomes. For each experiment, 2 μl of proteoliposomes was

placed in a 1-ml chamber containing recording solution (10 mM HEPES pH 7.4, 0.5 M KCl). The micropipettes used were borosilicate glass capillaries (Harvard Apparatus, Kent, United Kingdom) filled with recording solution. Membrane patches, obtained from unilamellar blisters from collapsing liposomes, were examined using standard patch-clamp technique. Single-channel currents were visualized with the Visual Patch 500 amplifier (Bio-logic), and recorded data were analyzed with Biotools and Analysis softwares (Bio-logic).

2.6. Comparative modeling

The amino acid sequence of *E. aerogenes* TolC and EefC was submitted to the FUGUE server (www-cryst.bioc.cam.ac.uk/fugue) [23] and searched against the HOMSTRAD data bank (www-cryst.bioc.cam.ac.uk/homstrad) [24] with default parameters. The structure-aided alignments of *E. aerogenes* TolC with *E. coli* TolC (PDB code 1EK9), and *E. aerogenes* EefC with *P. aeruginosa* OprM (PBD code 1WP1) were generated by FUGUE. These alignments were used to build models of *E. aerogenes* TolC and EefC using PDB Viewer.

3. Results

3.1. Purification of TolC and EefC

E. aerogenes TolC and EefC were produced as His$_6$-tagged recombinant proteins in *E. coli* BL21(DE3)pLysS. It is noteworthy that EefC contains a characteristic lipoprotein box with a conserved cysteine residue immediately downstream of an N-terminal signal sequence, *C*VSL [25]. Under physiological conditions, the signal sequence is cleaved off by the signal peptidase II, and the N-terminal cysteine residue is acylated to allow anchoring into the outer membrane. Studies with mutants of the lipoprotein OprM of *P. aeruginosa* showed that it is not needed for function [26,27]. Moreover, the presence of lipids could interfere with reconstitution studies through non-specific interactions, and may introduce further complications due to heterogeneity caused by incomplete processing or lipid-modification. For these reasons, we examined a lipid-deficient form of the EefC protein, in which the native sequence signal including C16 was replaced with the signal sequence of the periplasm-addressed protein azurin. Cellular fractionation analysis showed that EefC was correctly located in the outer membrane (data not shown). His$_6$-tagged TolC and EefC were extracted with 5% Triton X-100 from the membranes and purified by immobilized nickel affinity chromatography (INAC) as described [22]. Elution fractions from the INAC were pooled and analyzed by SDS-PAGE. When mixed with 0.1% SDS sample buffer and not boiled, TolC and EefC migrated at an apparent molecular mass greater than 100 kDa (Fig. 1, lanes −). These were replaced by bands corresponding to the TolC and EefC monomers when the same samples were heated for 5 min at 100 °C in the presence of 3% SDS (Fig. 1, lanes +) (note that similarly to that of TolC of *E. coli*, denatured TolC of *E. aerogenes* migrates as a doublet; both bands assemble to form a single high molecular weight species). This showed that purified TolC and EefC retained their trimeric native form. Western blot analysis also indicated that the samples were free of the outer membrane *E. coli* porins OmpA, OmpC and OmpF that could have interfered with subsequent *in vitro* membrane reconstitution (data not shown).
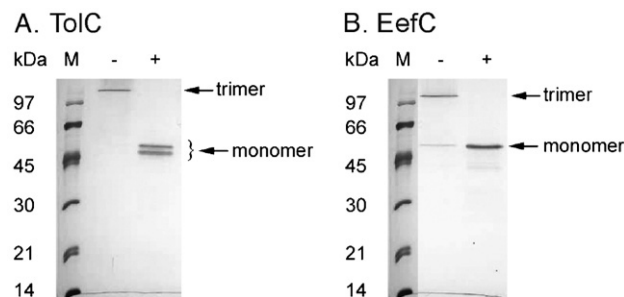

Fig. 1. Purification of *E. aerogenes* TolC and EefC. Elution fractions from affinity chromatography were pooled, heated at 100 °C in the presence of 3% SDS (+) or solubilized in 0.1% SDS without heating (−). The samples were analyzed by SDS-PAGE and the gels were silver-stained. The masses (in kDa) of the molecular mass markers (M) are indicated on the left side of each panel.

3.2. Three-dimensional models of TolC and EefC

To assist in the modeling of the *E. aerogenes* TolC and EefC structures, we first aligned their primary sequence with that of two homologs, TolC from *E. coli* and OprM from *P. aeruginosa*, whose three-dimensional structure is known [3,5]. TolC proteins from *E. aerogenes* and *E. coli* share 82% sequence identity with only one significant sequence gap in the extracellular loop 2, between the β-strands 4 and 5. Cell-surface-exposed loops are hypervariable regions [28] that are exploited by colicin E1 for import and bacteriophage TLS for binding, while dispensable for TolC efflux and secretion activities [29,30]. EefC has only 22 and 43% sequence identity with TolC from *E. coli* and OprM from *P. aeruginosa*, respectively. However, it is striking that structurally significant residues are well conserved and these determine a common overall fold [1,28]. At the proximal entrance of the tunnel, glycine residues are necessary for two tight turns between the helices H3 and H4, and H7 and H8. Small alanine and serine residues located at the interface of adjacent helices promote dense packing, which leads to tunnel tapering and proximal closure. Conserved proline residues accommodate the transitions between the α-helices of the tunnel domain and the β-strands of the channel domain. At the bottom of the β-strands, aromatic residues form a ring around the channel domain. The aromatic ring has been observed in all outer membrane proteins crystallized so far and may have an anchoring function. Conservation of the principle structural elements among TolC homologs has been demonstrated and suggests a common efflux mechanism [1].

Of particular interest are the residues at the proximal entrance of the tunnel. In the case of TolC from *E. coli*, a ring of six aspartate residues circles the periplasmic entrance of the tunnel (D371 and D374 in each of the three monomers). It has been shown that the aspartate ring is largely responsible for TolC's electrophysiological characteristics such as ion selectivity and pH-dependence [10,31]. These critical residues are conserved in TolC of *E. aerogenes* (D365 and D368), but are replaced by a serine (S401) and a threonine (T404) in EefC (Fig. 2). One can predict that the difference in charge distribution observed between *E. aerogenes* TolC and EefC may influence their
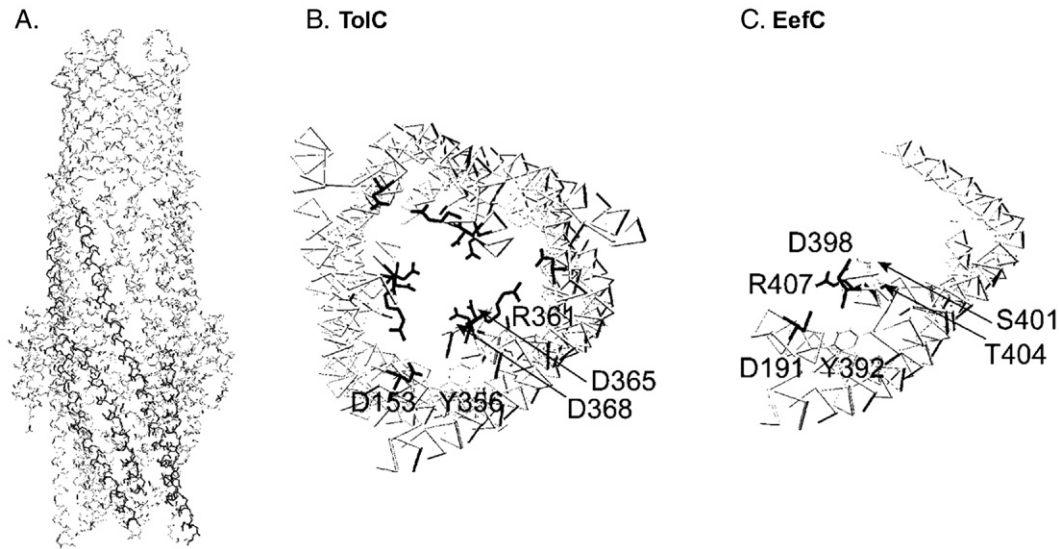
Fig. 2. Structural comparison of *E. aerogenes* outer membrane efflux proteins TolC and EefC. Structural models of *E. aerogenes* TolC and EefC were constructed using the three-dimensional structures of *E. coli* TolC (1EK9) and *P. aeruginosa* OprM (1WP1) as templates, respectively [3,5]. (A) Structural model of *E. aerogenes* TolC shown as a backbone. The four α-helices H3, H4, H7 and H8 of one of the three protomers are colored in black and are used magnified in (B) and (C). Bottom views of the closed periplasmic tunnel entrance of *E. aerogenes* TolC (B) and EefC (C) are shown. The residues lining the periplasmic entrance important for the constriction are labeled.

respective pore-forming properties *in vitro* and the selectivity of drug substrates *in vivo*.

3.3. Channel formation by TolC and EefC

In planar lipid bilayer experiments, TolC from *E. coli* showed an asymmetric channel conductance dependent on the transmembrane potential polarity (i.e., larger when the transmembrane potential was negative), with a mean single-channel conductance of about 80 pS in 1 M KCl [10].

Channel formation by *E. aerogenes* TolC and EefC was studied by single-channel conductance measurements after their reconstitution into giant liposomes and examination by the patch-clamp technique. Figs. 3 and 4 show selected current recordings from one excised patch of liposome containing TolC and EefC at +80 mV (panel A of both figures) and −80 mV (panel B of both figures) in 0.5 M KCl, respectively. From the current amplitude histograms of TolC, a mean conductance value of 70±6 pS was determined at positive potential (Fig. 3A) and a 73±9 pS value was found when the potential was negative (Fig. 3B). Single-channel conductance values of EefC were slightly higher than those of TolC: 89±5 pS when the potential was positive (Fig. 4A), and 96±5 pS when the potential was negative (Fig. 4B). The current recordings in Fig. 4 show the activity of multiple EefC channels in the same patch. Each current step has equivalent amplitude and corresponds to the closing of a single channel. The current flow through TolC and EefC increased with increasing voltages between 0 and ±120 mV, and the conductance values remained stable in this potential range (data not shown). At all potentials, EefC channels have about 30% higher conductance than TolC's. This can be explained by an enlarged tunnel entrance lined by serine and threonine

residues in EefC whose side chains are less bulky than those of aspartate residues in TolC.

Although the voltage-induced activation or inactivation of bacterial channels has not been demonstrated *in vivo* and may not have a physiological relevance, it can be used to compare related proteins. The voltage sensitivity of *E. aerogenes* TolC and EefC was further investigated by plotting macroscopic current versus voltage (*I/V*). Planar lipid bilayers containing a large number of reconstituted TolC or EefC were subjected to slow voltage ramps from −150 mV to ±150 mV. The *I/V* curves obtained for TolC and EefC channels were linear (i.e. ohmic) over the entire voltage range, indicating that no voltage-dependent openings or closures had occurred (see reference *I/V* curves in Figs. 5 and 6).

3.4. Ion selectivity

Previous studies on TolC from *E. coli* showed a preference for cations over anions, with a relative permeability ratio P_K/P_{Cl} of 16.5 in gradient of 30 mM to 300 mM KCl [10].

The selectivity of *E. aerogenes* TolC and EefC was investigated in multi-channel experiments. Proteins were added to the *cis* side of the membrane and the reversal potential (E_{rev}) was measured after establishing a 10-fold gradient across the lipid bilayer (0.1 M and 1 M KCl, the *cis* compartment was the most diluted). The relative permeability ratio P_{cation}/P_{anion} was calculated from E_{rev} by using the Goldman–Hodgkin–Katz equation. For TolC, calculated E_{rev} was 48±0 mV, with a relative permeability ratio P_K/P_{Cl} of 30. This means that TolC is 30-fold more permeable to potassium ions than to chloride ions. For EefC, calculated E_{rev} was 19.5 ± 1.5 mV, with a relative permeability ratio P_K/P_{Cl} of 2.9.
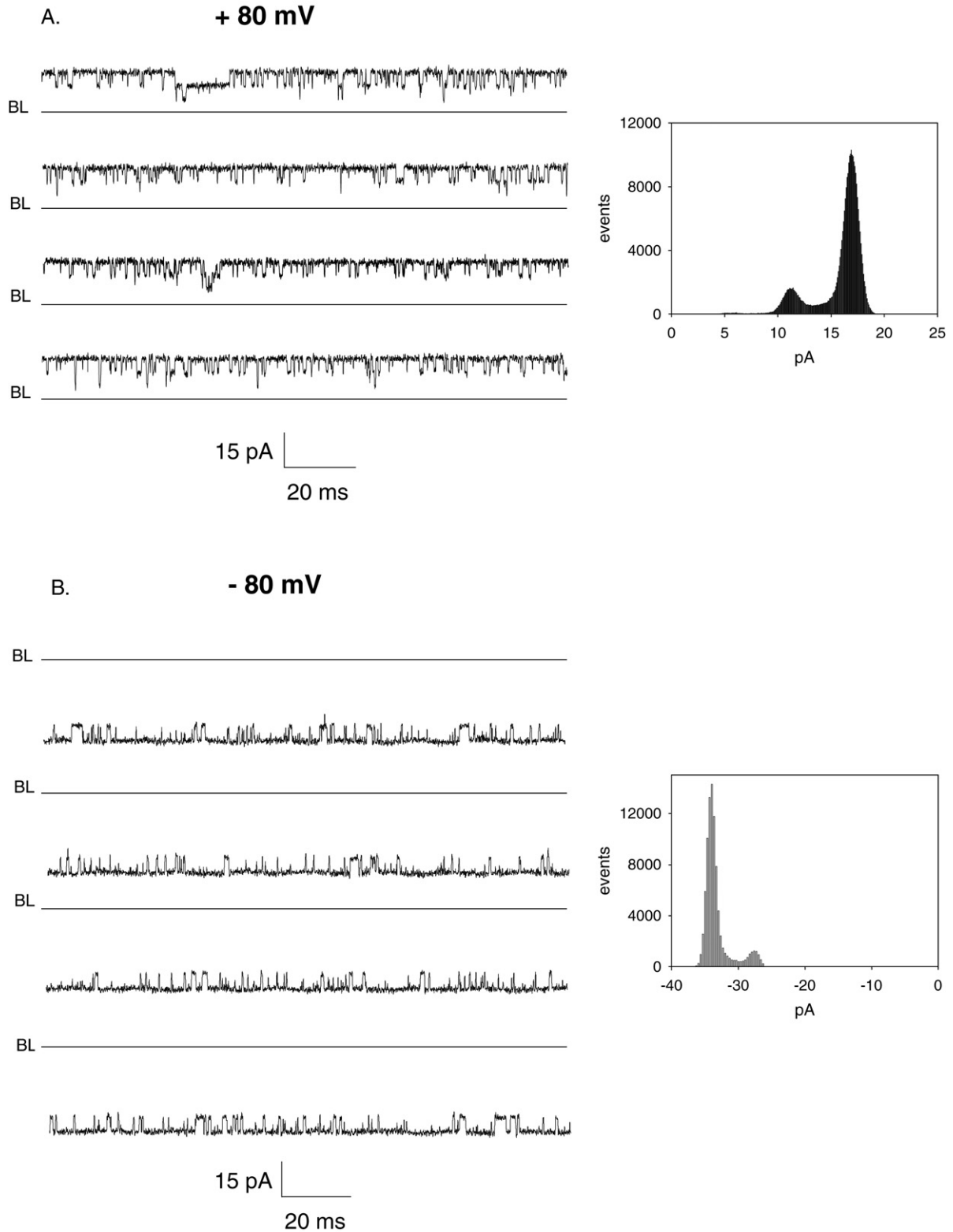
Fig. 3. Selected current recordings from *E. aerogenes* TolC ion channels and associated amplitude histograms. Giant liposomes of azolectin doped with TolC were studied by the patch-clamp method. Panel A, applied potential was +80 mV. Panel B, applied potential was −80 mV. BL represents the baseline at zero pA level. The buffer used was 10 mM HEPES (pH 7.4), 0.5 M KCl.
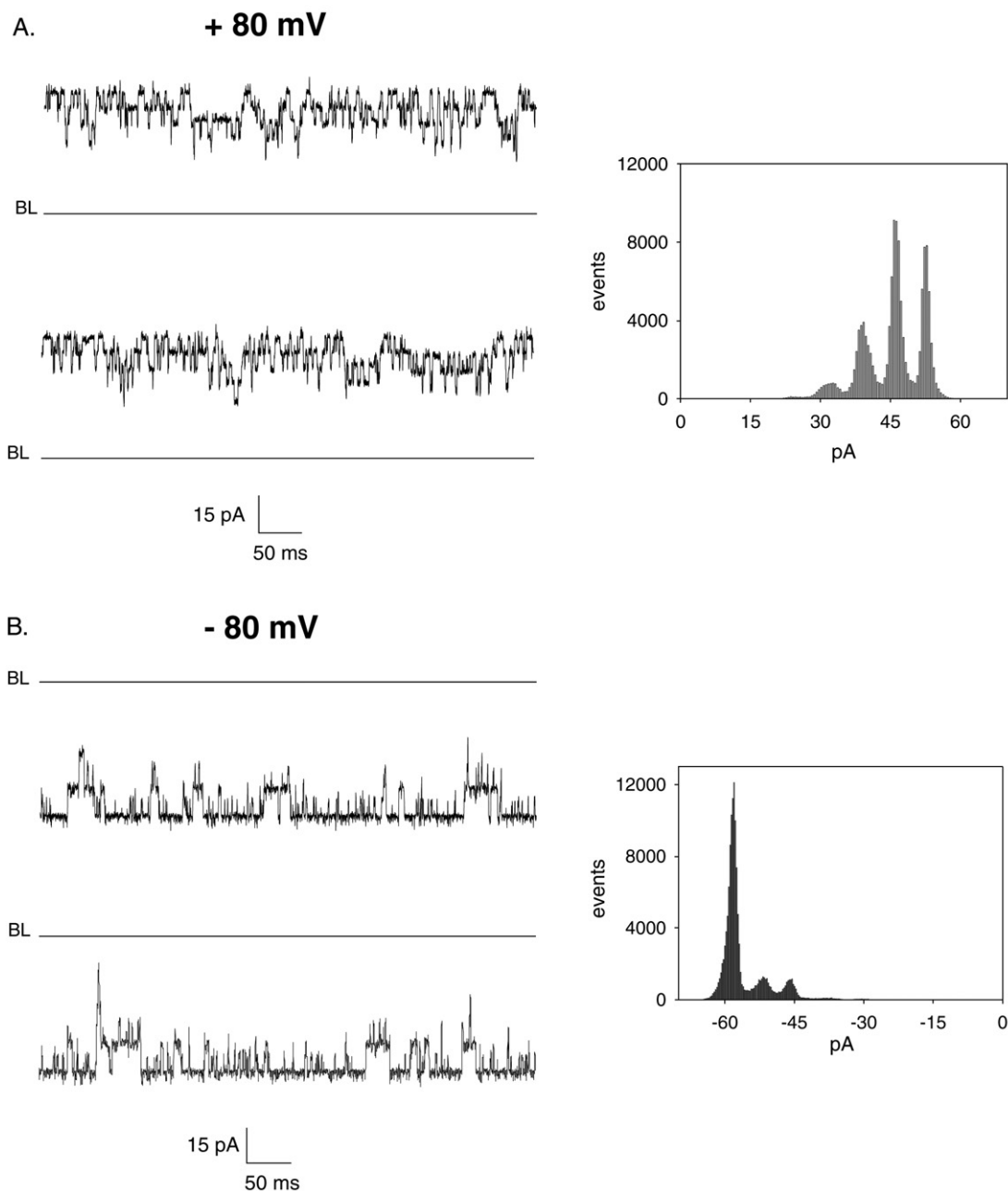
Fig. 4. Selected current recordings from *E. aerogenes* EefC ion channels and associated amplitude histograms. Same conditions as described in Fig. 3.

As observed in several previous studies, ion selectivity is critically affected by the charge constellation at the pore constriction [31–34]. The particular folding of the α-helices at the bottom of the tunnel determines the only constriction in *E. coli* TolC. The substitution of aspartate residues (D371 and D374) by alanine residues in this region clearly showed their role in the ion selectivity [1,31]. The proximal aspartate ring is conserved in *E. aerogenes* TolC (Fig. 2B), and both TolC from *E. coli* and *E. aerogenes* have similar ion selectivity. EefC was found approximately 10-fold lower than that of *E. aerogenes* TolC. This can be explained by the presence of uncharged serine and threonine residues (S401 and T404) located at the tunnel entrance (Fig. 2C). From the three-dimensional model of EefC, there are

also two charged residues (D398 and R407) that face the interior of the tunnel (Fig. 2C). Despite the absence of a strong electronegative ring, these remaining charges near the tunnel entrance can account for the weak cationic selectivity of EefC.

3.5. pH dependence

Another characteristic of the *E. coli* TolC channel-tunnel is its strong pH dependence [10]. The influence of pH on *E. aerogenes* TolC and EefC channels was investigated in multichannel experiments. The proteins were added to the *cis* side of a membrane bathed in 1 M KCl pH 7.4. After the *I*/*V* curves reached a stationary level, several microliters of 1 M citrate were

A. TolC

B. EefC
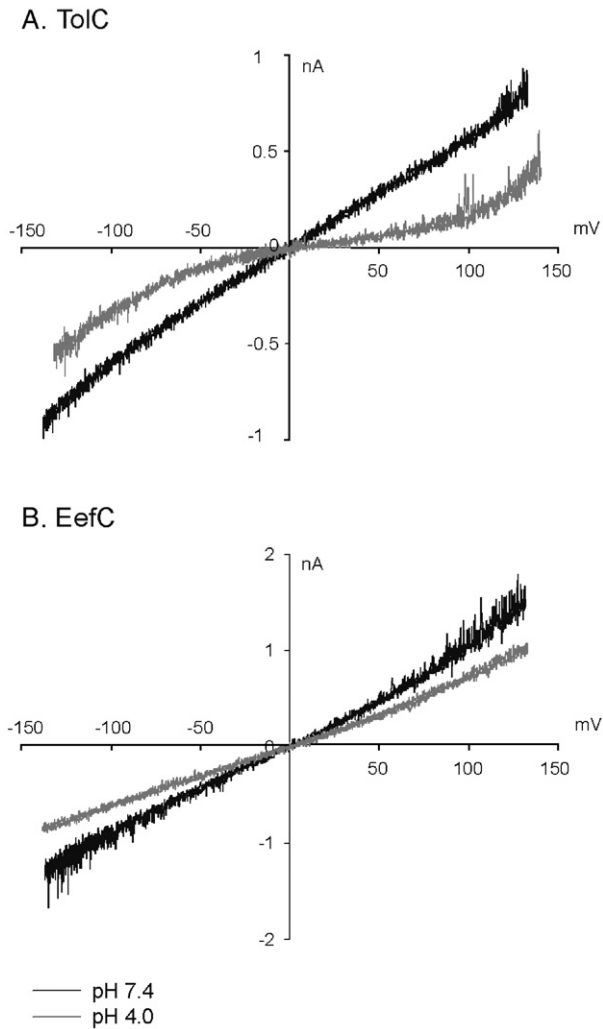

——— pH 7.4
——— pH 4.0

Fig. 5. Effect of acidic pH on *E. aerogenes* TolC and EefC macroscopic conductance. TolC (A) and EefC (B) were reconstituted into planar lipid bilayer of azolectin. Recordings were performed in 10 mM HEPES (pH 7.4), 0.5 M KCl. The pH was lowered by the addition of 1 M citrate under constant stirring. Voltage ramps: ±150 mV at 10 mV/s.

added to both sides of the membrane to lower the pH of the electrolyte solution to 4.0. As observed in Fig. 5, decreasing the pH caused a decrease in the current through both TolC and EefC channels, suggesting that the channel conductances were reduced. However, the shapes of two proteins' *I/V* curves at acidic pH are different. *E. aerogenes* TolC displayed a nonlinear *I/V* curve indicating that the channel conductance varied, dependent on the applied potential at pH 4 whereas the EefC channel conductance remained constant, independent of the potential as attested by its linear *I/V* curve. The variable conductance observed for *E. aerogenes* TolC is similar to what has been described previously at low pH for TolC from *E. coli* by Andersen and coworkers [10,31]. These authors suggested that at acidic pH, the disruption of hydrogen bonds involved in intra- and inter-protomer connections due to the protonation of the aspartate ring lining the constriction could allow the inner helices to move. These movements could constrict the channel entrance and consequently reduce the channel conductance.

The pH dependence nature of TolC from *E. coli* and *E. aerogenes* is in good agreement with the high sequence identity of these two proteins. In contrast, EefC, which shows a lower sequence identity with *E. coli* TolC, displayed different pH dependence. This result could be attributed to the absence of the aspartate ring in the EefC channel. However, the weaker pH dependence we observed for EefC could be due to the disruption of the hydrogen bonds of the intra-protomer connections that exist in this channel tunnel. In *E. coli* TolC, it has been stated that the protonation of E359 (inner helix 7) in a low pH environment would disrupt the interaction with Q136 (outer helix 3) [31]. Sequence alignment shows that this interaction is conserved in *E. aerogenes* TolC (E353-Q136) but not in EefC (M389-S174).

3.6. Channel inhibition by divalent cations

The single-channel conductance of *E. coli* TolC is severely inhibited by di- and trivalent cations introduced into the channel
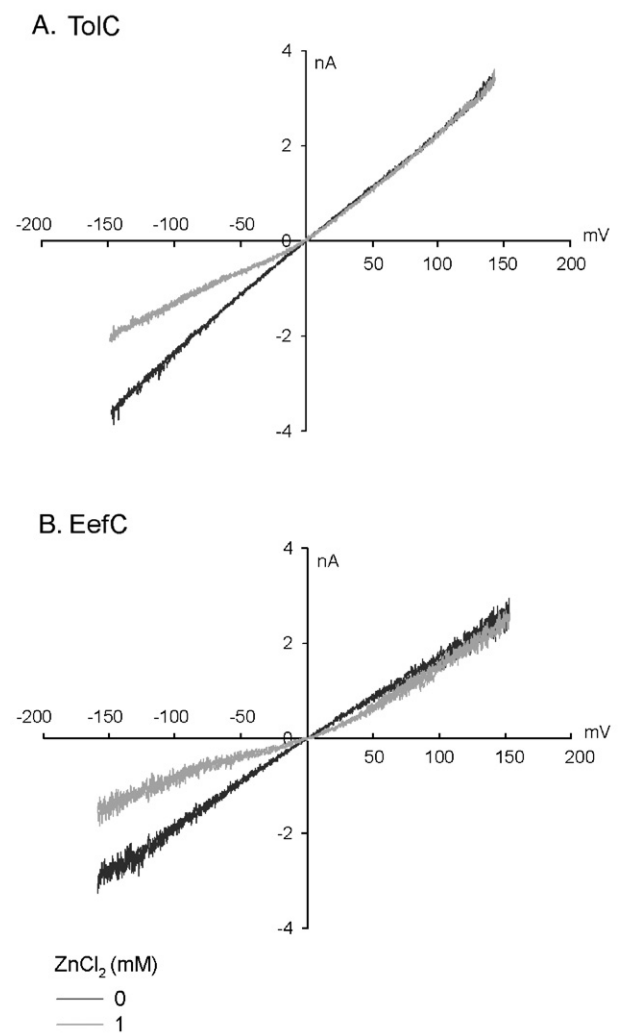
A. TolC

B. EefC

ZnCl₂ (mM)

——— 0
——— 1

Fig. 6. Zinc inhibition of *E. aerogenes* TolC and EefC macroscopic conductance. Composite *I/V* curves acquired for TolC (A) and EefC (B) in the absence or presence of ZnCl₂. ZnCl₂ was added at the *trans* side of the membrane under constant stirring. Voltage ramps: ±150 mV at 10 mV/s.

from the extracellular side. Of the divalent cations tested, Zn^{2+} and Co^{2+} were the most potent in blocking the channels [31].

Therefore, we examined the influence of Zn^{2+} on *E. aerogenes* TolC and EefC in multichannels experiments. There is evidence that the highly asymmetric TolC always inserts in the same orientation into the lipid membrane, with the channel domain first [10]. Consequently, the tunnel entrance of the inserted protein is always on the side to which the protein is added. We refer to this side as the *cis* or periplasmic side, in contrast to the *trans* or extracellular side. TolC and EefC were always added to the *cis* side of azolectin bilayers. Within a few hours, the macroscopic current flowing through multiple channels (up to fifty) increased to a steady level. $ZnCl_2$ caused a large decrease in both TolC and EefC channel conductance only when it was added to the *trans* side and in the presence of negative potentials (Fig. 6 and data not shown). Conductance was restored when the potential was changed to positive, suggesting that the voltage-induced blocking events were reversible. The requirement of a negative transmembrane potential for blocking is compatible with the cationic nature of $ZnCl_2$ and the general electronegative interior of the TolC family of channels —i.e. Zn^{2+} is 'pulled' into the channels. The negatively charged aspartate residues (D371 and D374) at the periplasmic entrance of TolC from *E. coli* constitute a binding site for di- and trivalent cations [31,35]. These residues are only accessible from the extracellular entrance. In addition, the flux of zinc cations, which have a hydrated radius of 3.53 Å, is hindered by both the small channel diameter and the strong electrostatic interactions at the periplasmic entrance. Sequence analysis of TolC and EefC from *E. aerogenes* indicates that residues D365 and D368 in TolC correspond to S401 and T404 in EefC (Fig. 2B and 2C). Thus, the ionic interactions are conserved in TolC but not in EefC. Furthermore, the low ion selectivity of EefC suggests that there is no other binding site for cations. Therefore, we hypothesized that the presence of serine and threonine residues at the periplasmic constriction of EefC generates enough local electronegativity to trap the zinc ions. However, we cannot rule out the influence of the nearby D398 (Fig. 2C).

4. Discussion

Structural and functional data of the outer membrane efflux proteins are necessary for a better understanding of the drug efflux mechanism and may eventually lead to rational design of inhibitory effectors. As evidenced by the increasing number of publications in this field, such data are now of importance as antibiotic efflux has a major influence on the MDR phenotypes of bacterial clinical isolates. Of particular interest is the ubiquity of the TolC family throughout Gram-negative bacteria, making it a potential target. In this study, we investigated the pore-forming properties of the outer membrane efflux proteins TolC and EefC from the nosocomial pathogen *E. aerogenes* and found that: (i) TolC and EefC exhibit single channel conductance of 70 pS and 90 pS in 0.5 M KCl, respectively. Channels are stable and do not show voltage-dependence. The low single channel conductance of *E. aerogenes* TolC and EefC

is similar to that of *E. coli* TolC [10], which suggests a common gated structure. (ii) Both TolC and EefC are cation-selective, with TolC much more so than EefC. The ion selectivity together with the constricted periplasmic tunnel entrance explain why large divalent cations can block TolC and EefC channels, as observed for *E. coli* TolC [31]. (iii) TolC and EefC channels are pH-dependent, with a tendency to close at low pH. For *E. coli* TolC, both the ion selectivity and pH-dependence stem for the presence of an aspartate ring (D371 and D374) located at the periplasmic entrance of the tunnel [31]. This feature is conserved in *E. aerogenes* TolC (D365 and D368) but not in EefC, which possesses a serine (S401) and a threonine (T404) at the corresponding positions. Nevertheless, it seems reasonable that there must be at least one acidic residue at the periplasmic entrance of EefC that accounts for the ion selectivity and pH-dependence, and that candidate is D398.

In the absence of a sequencing project of the *E. aerogenes* genome, we previously used complementation of *E. coli acrAB* or *tolC* mutants to identify drug efflux systems in this species. The tripartite AcrAB-TolC system was the first major identified efflux pump in *E. aerogenes* and was shown to contribute to MDR in clinical isolates [18]. Then we studied the *eefABC* locus, in which *eefC* encodes the outer membrane component [19]. Drug susceptibilities assays indicated that the expression of EefC conferred substantial resistance to antibiotics in both *E. coli* and *E. aerogenes tolC* mutants, suggesting that EefC can interact with the endogenous AcrAB pump to form a functional complex (M. Masi, unpublished results). The *eefABC* locus is silent under laboratory growth conditions, but it is transcribed in *hns* (H-NS, Histone like-Nucleoid Structuring protein) mutants and in spontaneous chloramphenicol-resistant mutants [19,36]. Furthermore, it is noteworthy that the drug resistance phenotypes caused by the overexpression of AcrAB-TolC or EefABC are quite different: AcrAB-TolC is involved in the efflux of various drugs including fluoroquinolone, tetracycline and macrolides families, whereas EefABC exhibits a more restricted substrate spectrum [18,19,36]. It is thought that the substrate specificity is mainly provided by the inner membrane component, but the charge distribution of the outer membrane component may facilitate or impede substrate movement across the channel. The different ion selectivity between *E. aerogenes* TolC and EefC observed *in vitro* might reflect the difference in drug selectivity between AcrAB-TolC and EefABC observed in vivo.

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