The channel-tunnel HI1462 of *Haemophilus influenzae* reveals differences to *Escherichia coli* TolC

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Efflux pumps play a major role in multidrug resistance of pathogenic bacteria. The TolC homologue HI1462 was identified as the single channel-tunnel in *Haemophilus influenzae* required to form a functional multidrug efflux pump. The outer-membrane protein was expressed in *Escherichia coli*, purified and reconstituted in black lipid membranes. It exhibited a comparatively small single-channel conductance of 43 pS in 1 M KCl and is the first known TolC homologue which is anion-selective. The HI1462 structure was modelled and an arginine residue lining the tunnel entrance was identified. The channel-tunnel of a mutant with the arginine substituted by an alanine residue was cation-selective and had a sevenfold higher single-channel conductance compared to wild-type. These results confirm that the arginine is responsible for anion selectivity and forms a salt bridge with a glutamate residue of the adjacent monomer, establishing a circular network, which keeps the tunnel entrance in a tightly closed conformation. In *in vivo* experiments, both the wild-type HI1462 and the mutant were able to substitute for *E. coli* TolC in the haemolysin secretion system, but not in the AcrAB/TolC multidrug efflux pump. The structure–function relationship of HI1462 is discussed in the context of the well-studied TolC channel-tunnel of *E. coli*.

INTRODUCTION

Channel-tunnel-dependent export systems contribute to the pathogenicity of various Gram-negative bacteria (Nikaido, 1994; Delepelaire, 2004). Channel-tunnels are pore-forming proteins located in the outer membrane, which interact with diverse inner-membrane complexes to form distinct export machinery (Andersen *et al.*, 2002a; Koronakis *et al.*, 2004). The type I secretion system is a channel-tunnel-dependent export system for proteins, such as toxins, S-layer proteins and diverse extracellular-acting enzymes. The paradigm for this export apparatus is the haemolysin secretion system of *Escherichia coli* (Wandersman & Delepelaire, 1990). In addition to protein secretion, channel-tunnels are also involved in export of noxious compounds, such as antibiotics, dyes, detergents, bile salts and heavy metals (Poole, 2005; Borges-Walmsley *et al.*, 2003). These channel-tunnel-dependent efflux pumps play a major role in establishing the resistance of most Gram-negative bacteria to these compounds, and are becoming an increasing problem for the clinical use of antibiotics.

Currently, the structures of three channel-tunnels are known (Koronakis *et al.*, 2000; Akama *et al.*, 2004; Federici *et al.*, 2005). The trimeric assembly forms a 140 Å (14 nm) long,

cannon-shaped structure. It is anchored in the outer membrane by a β -barrel domain and protrudes into the periplasm via a 100 Å (10 nm) long, α -helical tunnel domain, which assembles with inner-membrane complexes to form a continuous export pathway across the cell envelope. In contrast to the wide-open, extracellular entrance, the periplasmic entrance is almost closed, which explains the very low single-channel conductance of the best-characterized representative TolC of *E. coli* (Andersen *et al.*, 2002a). For TolC, the role of individual residues in electrophysiological characteristics and opening of the periplasmic entrance has been determined (Andersen *et al.*, 2002b, c). It can be shown that an iris-like outwards movement of the inward-folded tunnel helices is necessary to open the periplasmic entrance, allowing export of substrates (Eswaran *et al.*, 2003; Andersen *et al.*, 2002b).

In *Haemophilus influenzae*, a human pathogen responsible for significant morbidity and mortality in young children (Turk, 1984; Funkhouser *et al.*, 1991), a multidrug efflux pump has been identified, whose components are homologues of the *E. coli* AcrAB/TolC efflux pump (Sanchez *et al.*, 1997; Trepod & Mott, 2004). The AcrB-homologous HI0895 is a transporter of the resistance nodulation cell division family, which forms a complex with the AcrA-homologous adaptor or membrane fusion protein HI0894 (Sanchez *et al.*, 1997). This inner-membrane complex interacts with the TolC-homologous, outer-membrane channel-tunnel

Abbreviations: LDAO, lauryldimethylamine oxide; NTA, nitrilotriacetic acid.

HI1462 to assemble a functional multidrug efflux pump (Trepod & Mott, 2004). Disruption of any of the coding genes causes hypersusceptibility to antibiotic agents (Dean *et al.*, 2005; Sanchez *et al.*, 1997; Trepod & Mott, 2004). It can also be shown that a loss of pump repression by mutation or disruption of the AcrR repressor gene is the origin of increased resistance of several clinical *H. influenzae* isolates, illustrating the importance of this efflux pump (Dean *et al.*, 2005).

In this study, we have isolated the TolC-homologous HI1462 and characterized the biophysical properties of this pore-forming protein in lipid bilayer experiments. We present a computer model of the HI1462 channel-tunnel which explains its biophysical characteristics. Furthermore, we tested the ability of HI1462 to substitute the *E. coli* TolC in the AcrAB/TolC efflux pump and the HlyBD/TolC type I secretion system.

METHODS

Construction of HI1462 and TolC expression vectors. At first, the *HI1462* gene was cloned into the pAraJS.2 vector using *H. influenzae* Rd chromosomal DNA as template and two oligonucleotides, 5'-GGTGCATTGGTGGGAGCTCCCAATATTGGCGATTCTTATC-3' and 5'-CGGTTTCAGAAAAAGCAGCTCAGCCAGTAGATGCAGT-GAAAG-3', as primers (with restriction sites for *SacI* and *Bpu1102I*, respectively). This vector is a pET-12a-based plasmid, with insertion of a DNA cassette donating a His₁₀-tag sequence directly after the OmpT leader sequence and a more variable multicloning site (*SmaI*/*BamHI*/*NcoI*/*SacI*/*XhoI*/*BsrGI*/*Bpu1102I*). The cassette was constructed with oligonucleotides JS2_up (5'-TCGACGCATCATCACCATCAC-CATCACCATCACCACGGCGCCGAAGGCCGCCCGGGATCCAT-GGAGCTCGAGTGACAGC-3') and JS2_down (5'-AGTCGACAT-GTGAGCTCGAGGTACCTAGGGCCCCGCCGAAGCCGCGGCA-CCACTACCACCACCCTACCCTACTACTACGC-3'), leading to *SalI* and *Bpu1102I* overhangs, and ligated into an *SalI*/*Bpu1102I*-digested

pET12-a vector using T4 DNA ligase, resulting in p12JS2. Due to the tighter control of the araBAD promoter compared to the plac promoter of p12JS2, the initially mentioned promoter, together with *araC*, was amplified by PCR using the oligonucleotides 5'-GCGC-GAGGCAGCAGATCTATTCG-3' and 5'-CGAGCTCGGATCCATA-TGTAATTCCTCCTGTTAGCCC-3' as primers and pBAD/myc-His A (Invitrogen) as template. The plac promoter of p12JS2 was now replaced by araBAD using *BglII*/*NdeI* restriction sites resulting in pAraJS.2.

In the same way, the pAra21 vector was made by substituting the IPTG-inducible T7 promoter region of pET-21a (Novagen) with araBAD. Then, the *HI1462* gene, conjoined with the N-terminal His-tag, was cloned from pAraJS.2HI1462 into pAra21 using the primers 5'-GAATTACATATGCGGGCGAAAC-3' and 5'-GATTAATCCAAT-TTGATGAATTCAAATAATATCCCGCCATTG-3' (with restriction sites for *NdeI* and *EcoRI*, respectively). The resulting vector contained the *HI1462* gene with an N-terminal His₁₀-tag and a C-terminal His₆-tag under the control of an arabinose-inducible promoter.

The mutation of the arginine residue 396 to alanine was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) on pAra21HI1462biHis with the mutation primers 5'-GCGTATCCGA-ATTGGCCGAATGGTTAGTTGC-3' and 5'-GCAACTAACCATTCG-GCCAATTCGGATACGC-3', following the standard protocol.

tolC was cloned into pBAD/myc-His C using *E. coli* chromosomal DNA as template and the oligonucleotides 5'-CCACAAGGAATG-CTCATGAAGAAATTGC-3' and 5'-GTCGTCATCAGTTACGGAAT-TCGTTATGACCG-3' as primers (with restriction sites for *PagI* and *EcoRI*, respectively). All constructs were confirmed by DNA sequencing (Seqlab).

Bacterial strains and growth conditions. The *E. coli* strains and plasmids used in this study are listed in Table 1. The *tolC* knockout in AG100 and DC14 was performed according to the method of Datsenko & Wanner (2000). A TolC-KO_up primer (5'-CGCGCTA-AATACTGCTTCACCACAAGGAATGCAAATGAAGAAGTGTAG-GCTGGAGCTGCTTC-3'), a TolC-KO_down primer (5'-CCGTTA-CTGGTGTAGTGCGTGCGGATGTTTGCTGAACGACTGCATAT-GAATATCCTCCTTA-3') (underlined region binds to pKD3) and

Table 1. Strains and vectors

Strains	Genotype	Reference or source
AG100		Jellen-Ritter & Kern (2001)
AG100TC	<i>ΔtolC</i>	This laboratory
DC14	<i>ΔacrAB::kan</i>	Jellen-Ritter & Kern (2001)
DC14TC	<i>ΔtolC, ΔacrAB::kan</i>	This laboratory
BL21(DE3)Omp8	<i>ΔlamB ompF::Tn5 ΔompA ΔompC</i>	Prilipov <i>et al.</i> (1998)
Vectors		
pET12-a		Novagen
p12JS.2	pET12-a + JS.2-cass.	This laboratory
pET21-a		Novagen
pAra21a	pET21-a + <i>araCaraBAD</i>	This laboratory
pAraJS.2	pET12-a + JS.2-cass. + <i>araCaraBAD</i>	This laboratory
pAraJS.2HI1462	<i>HI1462</i>	This work
pAra21HI1462biHis	<i>HI1462</i> (N-terminal His ₁₀ - and C-terminal His ₆ -tags)	This work
pAra21HI1462R396A	<i>HI1462RA</i> (N-terminal His ₁₀ - and C-terminal His ₆ -tags)	This work
pBAD/myc-His C		Invitrogen
pBADTolC	<i>TolC</i> (C-terminal His ₆ -tag)	This work
pRSC2	Wild-type <i>hly</i> cluster: <i>hlyR, C, A, B, D; Cm^R</i>	Schulein <i>et al.</i> (1992)

pKD3 as template were used for PCR to produce a *tolC* knockout fragment. The loss of *tolC* was verified by colony PCR using primers that bind up- and downstream of *tolC*. The resulting strains were denoted AG100TC and DC14TC, respectively.

E. coli strains were grown at 37 °C with aeration in Luria–Bertani (LB) medium (Difco). When necessary, selective antibiotics were added.

Haemolysis was tested on blood-agar plates consisting of 25 ml LB agar supplemented with 0.75 ml defibrinated horse blood, spotted with 0.2 µl cell suspension (1×10^9 cells ml⁻¹) and incubated for 18 h at 37 °C. MIC tests were performed in 96-well plates by serial dilution of the tested antimicrobial agent in LB medium with 2×10^4 cells per well as the inoculum. The plates were incubated overnight at 37 °C and analysed photometrically after 24 h with an ELISA reader (Molecular Devices) at 600 nm.

Protein expression. *E. coli* BL21(DE3)Omp8 pAra21HI1462biHis was routinely grown at 37 °C with shaking (150 r.p.m.) in LB medium with ampicillin. Expression of HI1462 was induced by adding 0.02 % (w/v) arabinose at OD₆₅₀ 0.5–0.6. The culture was grown at 30 °C to prevent formation of inclusion bodies. After 3 h, cells were harvested by centrifugation (5000 g for 15 min) and washed twice in 10 mM Tris/HCl (pH 8.0). Cells were passed three times through a French pressure cell at 900 p.s.i. (6210 kPa). Unbroken cells were removed by centrifugation at 5000 g for 15 min. The cell envelope was obtained by centrifugation of the supernatant at 170 000 g for 60 min. The pellet was successively resuspended in 2 ml 1 % (v/v) Triton (twice), 1 % (v/v) lauryldimethylamine oxide (LDAO) and 2 % (v/v) LDAO followed by centrifugation at 170 000 g for 60 min, respectively. The 2 % (v/v) LDAO supernatant contained HI1462. For further purification, the supernatant was separated by FPLC using a MonoQ column (Pharmacia) and 0.5 % (v/v) LDAO 10 mM Tris (pH 8.0) as buffer. For elution of bound proteins, the KCl concentration in the buffer was raised continuously from 0 to 1 M. HI1462 eluted at 200 mM KCl. Finally, HI1462-containing fractions were applied to His-Select Ni-nitrilotriacetic acid (NTA) spin columns (Sigma).

SDS-PAGE and Western blotting. SDS-PAGE was performed according to the Laemmli gel system (Laemmli, 1970). The gels were stained with Coomassie brilliant blue or with silver stain (Blum *et al.*, 1987). Western blots were processed with the Bio-Rad Tank-Blot system with Protran nitrocellulose transfer membranes (Schleicher & Schuell BioScience) by applying a constant current of 350 mA for 30 min. The ECL Western Blotting Detection kit (Amersham Pharmacia Biotech) was used to detect binding of the Anti-His antibody according to the manufacturer's instructions.

Lipid bilayer experiments. The methods used for the lipid bilayer experiments have been described in detail by Benz *et al.* (1978). Black lipid bilayer membranes were obtained from 1 % (w/v) diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane. The temperature was maintained at 20 °C during all experiments. Zero-current membrane potentials were measured by establishing a salt gradient across membranes containing 100–200 channels, as described by Benz *et al.* (1985).

Protein modelling. The HI1462 structure was modelled using the Homology module of the InsightII software package (Accelrys) based on the structure of the closest homologous channel-tunnel, OprM of *Pseudomonas aeruginosa* (Akama *et al.*, 2004). A refinement of the structure was performed using the CharmM module of the InsightII software package.

RESULTS

Purification of HI1462

In a first attempt, we tried to purify the N-terminal, His-tagged version of HI1462 expressed by pAraJS.2HI1462 in *E. coli* BL21(DE3)Omp8. The protein was extracted from the cell membrane by 2 % (v/v) LDAO. However, purification by affinity chromatography revealed that binding of HI1462 to the Ni-NTA column was weak and the majority of the protein was already eluted when washing the column with 50 mM imidazole. HI1462 eluted by 250 mM imidazole showed a single band in SDS-PAGE but reconstitution in the very sensitive planar lipid bilayer setup revealed that the sample contained other pore-forming proteins, which were also observed when adding detergent extracts of BL21(DE3)Omp8 cells not expressing HI1462. In order to obtain purer protein samples, we constructed a double His-tagged version of HI1462. The protein bound much tighter to the Ni-NTA column, which made it possible to wash the column extensively, eliminating non-specifically bound protein. Elution of HI1462 by 500 mM imidazole (pH 8.0) resulted in highly pure protein (Fig. 1). When incubated at 100 °C prior to SDS-PAGE, the protein ran at 48 kDa, concordantly with the calculated value for the HI1462 monomer. Without a denaturing step, the band shifted to ~110 kDa, corresponding to HI1462 trimer. By Western blot analysis, we could clearly confirm that both bands represented HI1462.

Fig. 1. SDS-PAGE (10 % polyacrylamide) of His-tagged HI1462 and HI1462RA samples, performed according to the method of Laemmli and silver-stained (Laemmli, 1970; Blum *et al.*, 1987). Purified protein samples (10 µl) were treated for 10 min at 100 °C or at room temperature (RT) before loading.

Reconstitution of HI1462 in black lipid membranes

For biophysical characterization of purified HI1462, protein was reconstituted in black lipid membranes. After addition of 2 ng protein to the aqueous phase, a stepwise increase of conductance was observed; each step corresponding to membrane-insertion of a single HI1462 trimer (Fig. 2). We assumed that the double His-tag on both ends of the protein had no influence on the single-channel conductance. It is very unlikely that the N-terminal His-tag, which was located at the 37-residue-long tail outside the conduit structure, had any influence on the channel-forming characteristics. The double-tagged version led to the same conductance steps as the single-tagged version, therefore, we concluded that the C-terminal His-tag, which was closer to the tunnel structure but still not part of it, also had no influence. The single-channel conductance in 1 M KCl was 43 pS. Measured in potassium acetate and LiCl, the single-channel conductance was 21 and 39 pS, respectively. This means that exchange of the cation had only a minor effect, while exchange of the anion led to a major decrease. This is the first hint that HI1462 is an anion-specific channel. Ion-specificity was quantified by zero-current potential measurements in the presence of salt gradients. A fivefold KCl gradient across a membrane with reconstituted HI1462 led to a membrane potential of 11.6 mV, negative on the diluted side. Analysis using the Goldman–Hodgkin–Katz equation resulted in a permeability ratio (P_K/P_{Cl}) of 0.5. Thus, the HI1462 channel

is anion-selective, as suggested by the single-channel recordings.

Measuring the single-channel conductance in lower KCl concentrations revealed a linear dependence between 1 and 0.3 M KCl. A decrease to 0.1 M KCl resulted in almost no further reduction of the single-channel conductance. The single-channel conductance recorded in 3 M KCl showed major flickering, and conductance steps varied between 60 and 100 pS, with a mean value of ~81 pS.

Further biophysical characterization of HI1462 channels revealed that the pores were not voltage-dependent in a range between +100 and –100 mV, meaning that the conductance remained unchanged, and no opening or closing was observed. Unlike TolC of *E. coli* HI1462, channels could not be blocked by the divalent cation Zn^{2+} .

Model of the HI1462 structure explains its biophysical characteristics

The biophysical analysis revealed that HI1462 is anion-specific. This is a major difference to TolC of *E. coli*, which is highly selective for cations (Andersen *et al.*, 2002a). To obtain a structural insight at the molecular level, we modelled the structure of HI1462. At present, the structures of three channel-tunnels are known: TolC of *E. coli*, OprM of *P. aeruginosa* and VceC of *Vibrio cholerae* (Koronakis *et al.*, 2000; Akama *et al.*, 2004; Federici *et al.*, 2005). A sequence alignment of the HI1462 sequence showed the best homology with the sequences of OprM (data not shown). Therefore, we used OprM as a template to model the HI1462 structure (Fig. 3a). Of particular interest are residues at the periplasmic end of the tunnel domain. It is known from *E. coli* TolC that residues lining the periplasmic entrance have a major effect on the electrophysiological behaviour of the channel-tunnel. Looking at the HI1462 model, there are valine residues (Val400) located on the second helical turn of the most inwardly directed helix, facing the lumen of the periplasmic entrance (Fig. 3b). These hydrophobic residues might be responsible for the low single-channel conductance observed for HI1462. Below this hydrophobic region, there are three charged residues per monomer, Glu394, Arg396 and Glu397, whereas the arginine residues are nearest to the lumen and are responsible for the electropositive lining of the periplasmic entrance. We suppose that these arginine residues are the origin of the anion-specificity of HI1462.

Fig. 2. Single-channel recording of HI1462 wild-type pores. Channel-forming activity was measured with lipid bilayer membranes made from a 1 % (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids) in *n*-decane (Benz *et al.*, 1978). The aqueous phase contained 1 M KCl and 2 ng protein ml^{–1}. The applied membrane potential was 50 mV and the temperature was 20 °C. The single-channel recordings were performed using Ag/AgCl electrodes (with salt bridges) connected in series to a voltage source and a Keithley 427 current amplifier. The amplified signal was monitored on a strip chart recorder.

A single-point mutation at the periplasmic entrance changes the biophysical properties of HI1462

To prove the dominant role of Arg396 in the electrophysiological properties of HI1462, we substituted the amino acid with alanine. The resulting mutant HI1462RA was purified and electrophysiologically characterized in the same way as the HI1462 wild-type. The single-channel conductance in 1 M KCl was 282 pS, which was almost sevenfold higher than that of HI1462 wild-type, showing

Fig. 3. (A) Structural model of an HI1462 trimer shown as a backbone representation. One monomer is coloured. The backbone of the peptide chain outside of the tunnel domain is shown in black. The α -helices and β -strands forming the channel-tunnel are highlighted in the same colour scheme as in (B). Tunnel entrance of HI1462 (B) and of TolC (C) seen from the periplasmic side. Helices of one of the three monomers are coloured green (H3, outer coiled coil), blue (H4, outer), yellow (H7, inner coiled-coil) and red (H8, inner). Important residues are shown in detail. Depictions were generated using WebLab Viewer (Accelrys).

that the amino acid substitution had, in fact, a massive effect on the single-channel conductance. In contrast to HI1462 wild-type, there was an almost linear dependency of the single-channel conductance with a KCl concentration of 0.03–1 M. At a higher salt concentration, it was not possible to measure single insertion events. Here, the protein-induced conductance increase was very noisy and no defined step could be identified. The single-channel conductance measured in LiCl and potassium acetate gave the first hint that the ion selectivity was also changed in HI1462RA. In contrast to HI1462 wild-type, the change of cation from K^+ to Li^+ led to a massive decrease in single-channel conductance, whereas the single-channel conductance measured in potassium acetate was similar to that measured in KCl (Table 2). A quantitative determination of ion selectivity by zero-current measurements supported the assumption that exchange of Arg396 by alanine reversed ion selectivity. The permeability ratio (P_K/P_{Cl}) was 8.6, showing that HI1462RA was cation-selective.

However, beside pores with a single-channel conductance of ~ 280 pS, we also observed a second type of pore for HI1462RA with a single-channel conductance between 1.9 and 2.1 nS in 1 M KCl. The pores were rarely observed and their lifetime was very short. They closed immediately and adopted a conformation with a single-channel conductance of ~ 280 pS corresponding to the small pores (Fig. 4). This observation let us conclude that both pore types can be attributed to the HI1462RA protein adopting two different conformations, and not to another co-purified, pore-forming protein. In lower salt concentrations, the higher conductance state was not observed.

HI1462 is able to substitute *E. coli* TolC in the haemolysin secretion apparatus but not in a multidrug efflux pump

Channel-tunnels are involved in diverse export processes. We tested if HI1462 can substitute *E. coli* TolC as an outer-membrane component of the type I haemolysin secretion system HlyBD/TolC, and of the multidrug efflux pump AcrAB/TolC. Haemolysin secretion was tested on blood agar plates with cells harbouring pRSC2 encoding the haemolysin operon (Fig. 5). Channel-tunnels TolC and

Table 2. Single-channel conductance of HI1462 and HI1462RA in different electrolytes and at different electrolyte concentrations

Each single-channel conductance value is based on at least 80 insertion events. The applied voltage was 20 mV and the temperature was 20 °C. NM, Not measurable.

Electrolyte	Concentration (M)	Conductance (pS)	
		HI1462	HI1462RA
KCl	3	81	NM
	1	43	282
	0.3	13	89
	0.15	—	45
	0.1	12	34
	0.03	NM	11
LiCl	1	39	122
Potassium acetate	1	21	269

Fig. 4. Single-channel recording of HI1462RA mutant pores. The aqueous phase contained 1 M KCl and 2 ng protein ml⁻¹. The applied membrane potential was 20 mV and the temperature was 20 °C.

HI1462 were expressed from an arabinose-inducible plasmid in the *tolC*-deficient strain AG100TC. An arabinose concentration of 0.1 % (w/v) was chosen for induction of channel-tunnels because it resulted in the same zone of clearance as AG100 cells expressing TolC from the chromosome. Cells lacking TolC or HI1462 had a small zone of clearance due to haemolysin release by cell lysis. Interestingly, cells expressing HI1462 wild-type, as well as the HI1462 mutant, were able to secrete haemolysin. However, the halo was much smaller compared to cells expressing TolC. When HI1462 or TolC were induced by higher arabinose concentration, the size of the halo increased,

Fig. 5. Test of haemolysin secretion on blood agar plates. All cells contained pRSC2 encoding the haemolysin operon. *E. coli* AG100 cells were used as a positive control with TolC expressed from the chromosome (TolC^{chrom}), and the TolC-deficient strain AG100TC harbouring the pBAD vector as a negative control (Δ TolC). Channel-tunnels TolC, HI1462 or HI1462RA were expressed from an arabinose-inducible plasmid in AG100TC cells. Plates were inoculated with 2×10^5 cells and grown for 18 h at 37 °C. Bar, 1 cm.

but cells expressing HI1462 always had a smaller zone of clearance.

To test if HI1462 is also able to substitute TolC in the multidrug efflux pump AcrAB/TolC, we determined the MIC of *tolC*-deficient AG100TC cells expressing HI1462 variants. Using TolC expressed from a plasmid-encoded, arabinose-inducible gene, we have determined the arabinose concentration necessary to reveal MIC values, which correspond to that of wild-type strain AG100. AG100TC cells expressing HI1462 or HI1462RA induced by the same arabinose concentration revealed MIC values which were indistinguishable from those of AG100TC cells expressing no channel-tunnel (Table 3). Therefore, we conclude that HI1462 is not able to form a functional, or at least an effective, efflux pump with the *E. coli* AcrAB complex.

DISCUSSION

According to a phylogenetic analysis of membrane transport systems, *H. influenzae* possesses six predicted multidrug efflux pumps (Paulsen *et al.*, 2000). Out of these, AcrAB/HI1462 has been characterized as the primary efflux pump, exporting a broad variety of toxic compounds (Sanchez *et al.*, 1997; Trepod & Mott, 2004). A decreased susceptibility of clinical isolates due to overexpression of efflux pump genes has confirmed the role of this efflux pump as a major determinant for drug resistance of *H. influenzae* (Dean *et al.*, 2005). Analysis of the chromosome has revealed that HI1462 is the only functional channel-tunnel in *H. influenzae*. A phylogenetic analysis separates the channel-tunnel family into three groups, corresponding to the export processes in which the proteins are involved: protein secretion, drug efflux and cation efflux (Andersen *et al.*, 2000). HI1462-like OprM of *P. aeruginosa* belongs to the group of channel-tunnels involved in drug efflux, whereas TolC of *E. coli* is a representative of the channel-tunnels involved in protein secretion. HI1462 reconstituted in planar lipid membranes revealed a single-channel conductance of 43 pS in 1 M KCl, almost half that of TolC, but 20-fold smaller than that of OprM (Andersen *et al.*, 2002a; Wong *et al.*, 2001). The low single-channel conductance of HI1462 accords well with

Table 3. Susceptibility of strains depending on the channel-tunnel

Abbreviations: VANC, vancomycin; ERM, erythromycin; R6G, rhodamine 6G. All strains were induced with 0.01 % arabinose.

Strains and plasmids	MIC (μ g ml ⁻¹)		
	VANC	ERM	R6G
AG100 pBad	> 12	> 50	> 1000
AG100TC pBadTolC	> 12	> 50	1000
AG100TC pAra	> 12	3-13	3-9
AG100TC pAra21HI1462biHis	> 12	3-13	3-9
AG100TC pAra21HI1462biHisRA	> 12	3-13	3-9

our modelled structure (Fig. 3). The periplasmic entrance of HI1462 is lined by three valine residues (Val400) forming a hydrophobic constriction, which is most likely responsible for the lower single-channel conductance of HI1462 compared to that of TolC. In TolC, there is an aspartate at the corresponding position (Asp374). Together with a second aspartate (Asp371), which is located one helical turn closer to the periplasmic entrance, it is responsible for the cation selectivity of TolC (Andersen *et al.*, 2002c). In HI1462, the second central position is also covered by a negatively charged residue (Glu397). However, our measurements revealed that HI1462 is the first known TolC homologue which is anion-selective. This also explains why divalent cations do not block the channel as observed for TolC (Andersen *et al.*, 2002c). We showed that the anion selectivity originates from the Arg396, which also lines the tunnel entrance. The dominant role of Arg396 was investigated in the mutant HI1462RA. Substitution of arginine by alanine reversed the ion selectivity of the HI1462 channel, implying that the tunnel entrance became electronegative due to the glutamate residues at positions 397 and 394 (Fig. 3). Additionally, the HI1462RA mutant had an increased single-channel conductance. However, the almost sevenfold higher value (282 pS), compared to wild-type, cannot be explained solely by the absence of the bulky arginine residues. Compared with the single-channel conductance of TolC (80 pS), which has no arginine residue at the corresponding position, the HI1462RA entrance needs to be opened further. When looking at the HI1462 model, it becomes evident that Arg396 is involved in stabilization of the closed conformation of the HI1462 tunnel entrance. Arg396 is in close proximity to Glu397 of the adjacent monomer, allowing the formation of a circular network of salt bridges, which keeps the inward-folded helices in position. The absence of these connections in HI1462RA might allow the inner helices to move outwards, explaining the higher single-channel conductance. Interestingly, these were not the only conductance steps seen for HI1462RA. We also observed insertion events with a six- to sevenfold higher single-channel conductance, which could be undoubtedly assigned to the HI1462RA mutant protein. This high conductance level was unstable and observed only rarely at high salt concentrations. We assume that the lack of the circular network allows the tunnel entrance to temporarily adopt a fully open state. At lower salt concentrations, the high conductance state was absent. This could be explained by additional ionic interactions which keep the tunnel entrance in a narrow conformation. At high ionic strength, these interactions are destabilized, allowing transition into the fully open state, whereas at lower salt concentrations, they are stable enough to keep the entrance in the narrow conformation. For TolC, it is known that the open state can be achieved by disturbance of a circular network of hydrogen bonds and salt bridges close to the periplasmic entrance (Andersen *et al.*, 2002b). Sequence alignment shows that the aspartate (Asp153 in TolC) located at the outer coiled coil, which is central to the formation of the circular network, is not conserved in HI1462, which

possesses a serine (Ser190) at the corresponding position. Therefore, it seems reasonable that there must be other connections, which keep the channel-tunnel in a closed conformation, to prevent unwanted influx or efflux through a wide-open channel-tunnel in the outer membrane. The electrophysiological characterization of the HI1462RA mutant shows that the salt bridge, which can be formed between Arg396 and Glu397 according to the modelled HI1462 structure, is most likely responsible for keeping the tunnel entrance in a tightly closed conformation.

It is surprising that OprM of *P. aeruginosa*, which shows a higher homology to HI1462 than TolC, has a single-channel conductance of 850 pS in 1 M KCl (Wong *et al.*, 2001), which is almost 20-fold higher than that of HI1462. However, the high single-channel conductance seems to conflict with the solved crystal structure of OprM, which shows a nearly closed periplasmic entrance (Akama *et al.*, 2004). It should be mentioned that the reconstituted channels are not stable and appear to form substates (Wong *et al.*, 2001), a characteristic also observed for the open state of TolC (Andersen *et al.*, 2002b). Additionally, the pore-forming activity of OprM in planar lipid bilayers is very low compared to other reconstituted pore-forming proteins (R. Benz, personal communication), which lets us assume that only a minority of the OprM molecules reconstitute as pores in a transient open state, whereas the majority of the molecules insert into the membrane as closed pores, which allows no passage of ions and cannot therefore be detected by electrophysiological measurements.

The genomic organization of the multidrug efflux pump in *H. influenzae* resembles that of *E. coli* to the extent that the gene encoding the channel-tunnel is not linked to those encoding the proteins of the inner-membrane complex. Among the six predicted multidrug efflux pumps in *H. influenzae*, there is another export mechanism coded by a gene for the membrane fusion protein EmrA (HI0898) and a transporter of the major facilitator superfamily EmrB (HI0897), which also needs a channel-tunnel protein to assemble a functional efflux pump according to the known homologues in *E. coli* (Lomovskaya & Lewis, 1992). Therefore, the sole channel-tunnel in *H. influenzae* HI1462 needs to be compatible with two different inner-membrane complexes. This is in accordance with TolC of *E. coli*, which is known to interact with at least eight different inner-membrane complexes (Andersen, 2003). Among these is the HlyBD complex, which with TolC forms a secretion apparatus for haemolysin (Wandersman & Delepelaire, 1990). Genes encoding a type I secretion system are absent in *H. influenzae*. Therefore, it is surprising that HI1462 can substitute for TolC in the *E. coli* protein secretion apparatus. The secretion was not as efficient as that of the wild-type system, which suggests either that the amount of HI1462 is lower compared to TolC, or that the two proteins HlyD and HI1462 are not fully compatible. By higher induction of HI1462, it was possible to increase the amount of secreted haemolysin. However, we never reached the maximal

secretion levels observed with cells expressing TolC. Therefore, reduced haemolysin secretion is probably caused by compatibility problems between HlyD and HI1462 in the hybrid system. A slightly bigger halo, observed for the hybrid system including the HI1462RA mutant, might lead to the supposition that facilitated opening of the tunnel entrance by interaction with HlyD plays a role in enhanced secretion. Other hybrid, type I secretion systems have exchanged the outer-membrane component with highly homologous channel-tunnels, e.g. TolC of *E. coli*, HasF of *Serratia marcescens*, and PrtF of *Erwinia chrysanthemii*. They can be interchanged between the different secretion systems without loss of functional secretion (Binet & Wandersman, 1996; Letoffe *et al.*, 1994; Akatsuka *et al.*, 1997). Recently, it has been shown that RaxC, a channel-tunnel of the plant pathogen *Xanthomonas oryzae* pv. *Oryzae*, complemented an *E. coli* tolC mutant in a type I secretion system (da Silva *et al.*, 2004). However, RaxC is also a channel-tunnel belonging to the protein secretion family. Thus, HI1462 is the first example of a channel-tunnel belonging to the drug efflux family which is able to act as an outer-membrane component for a type I secretion system.

Substitution of TolC by HI1462 as an outer-membrane component of the *E. coli* AcrAB efflux pump did not restore resistance. Similar results have been observed when trying to complement TolC by OprM of *P. aeruginosa* (Tikhonova *et al.*, 2002). It should be mentioned that MIC values are generally much lower for *H. influenzae* than for *E. coli* (Trepod & Mott, 2004). If HI1462 is responsible for the less efficient efflux pump of *H. influenzae*, the possibility cannot be excluded that the hybrid efflux pump AcrAB/HI1462 in *E. coli* is functional but does not give rise to the same level of resistance as the wild-type efflux pump. However, there are no reports in the literature which point towards the channel-tunnel as a rate-limiting factor for the efflux. Therefore, we conclude that HI1462 is not compatible with the inner-membrane complex of *E. coli*. This means that interaction between the inner-membrane complexes of drug efflux pumps and the corresponding channel-tunnels is more specific than the interaction of the inner and outer components of type I secretion systems. Further research is needed to understand these interactions at the molecular level. This is essential for developing drugs that inhibit these export mechanisms and disarm harmful bacteria.

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