Crystal structures of the OmpF porin: function in a colicin translocon

Eiki Yamashita^{1,3}, Mariya V Zhalnina¹,
Stanislav D Zakharov^{1,2}, Onkar Sharma¹
and William A Cramer^{1,*}

¹Department of Biological Sciences, Purdue University, West Lafayette, IN, USA and ²Institute of Basic Problems of Biology, Russian Academy of Sciences, Puschino, Moscow Region, Russian Federation

The OmpF porin in the *Escherichia coli* outer membrane (OM) is required for the cytotoxic action of group A colicins, which are proposed to insert their translocation and active domains through OmpF pores. A crystal structure was sought of OmpF with an inserted colicin segment. A 1.6 Å OmpF structure, obtained from crystals formed in 1 M Mg²⁺, has one Mg²⁺ bound in the selectivity filter between Asp113 and Glu117 of loop 3. Co-crystallization of OmpF with the unfolded 83 residue glycine-rich N-terminal segment of colicin E3 (T83) that occludes OmpF ion channels yielded a 3.0 Å structure with inserted T83, which was obtained without Mg²⁺ as was T83 binding to OmpF. The incremental electron density could be modelled as an extended poly-glycine peptide of at least seven residues. It overlapped the Mg²⁺ binding site obtained without T83, explaining the absence of peptide binding in the presence of Mg²⁺. Involvement of OmpF in colicin passage through the OM was further documented by immuno-extraction of an OM complex, the colicin translocon, consisting of colicin E3, BtuB and OmpF.

The EMBO Journal (2008) 27, 2171–2180. doi:10.1038/emboj.2008.137; Published online 17 July 2008

Subject Categories: membranes & transport; structural biology

Keywords: colicin E3; intrinsically disordered protein; OmpF porin; receptor BtuB; tol system

Introduction

Proteins in the outer membrane (OM) of Gram-negative bacteria such as *Escherichia coli* contribute to a permeability barrier that protects the cell from harmful solutes, but allow the import of small water-soluble metabolites whose size is limited by non-specific diffusion through these porins (Nikaido and Vaara, 1985; Bredin *et al*, 2003; Nikaido, 2003). Many OM proteins also function as receptors for phage and colicins (Cascales *et al*, 2007). The 551 residue

rRNase colicin E3, whose insertion into the OmpF porin is a subject of this study, binds tightly ($K_d < 10^{-9}$ M) to the BtuB receptor through its 135 coiled-coil central domain (Kurisu *et al*, 2003), exerts its cytotoxic effect through the endoribonucleolytic action of its 96 residue C-terminal domain (Ohno-Iwashita and Imahori, 1980), which is translocated into the cytoplasm and uses its N-terminal translocation domain to interact with its putative receptor-translocator, OmpF (Zakharov *et al*, 2004). The C-terminal catalytic domain is probably freed from bound R-domain by proteolysis between C- and R-domains, as shown for the DNase colicin E7 (Shi *et al*, 2005) or E2 (Sharma *et al*, 2007), where the cleavage occurs at Arg447 and Arg452, respectively. Preliminary evidence for a proteolysis site in a similar region of the rRNase colicin E3 has been obtained (O Sharma and WA Cramer, unpublished data).

OmpF porin, one of the most abundant proteins ($\geq 10^5$ copies per cell) in the OM (Nikaido, 2003), was the first integral membrane protein for which crystals could be obtained that diffracted to better than 4 Å (Garavito and Rosenbusch, 1980). OmpF is a symmetric trimer, consisting of three copies of a 340-residue monomeric unit (Cowan *et al*, 1992) that functions as a weakly cation (Benz, 1988) and size-selective filter with an exclusion limit of approximately 0.6 kDa for hydrophilic solutes (Nikaido, 2003).

High-resolution (2.2–2.4 Å) crystal structures (Cowan *et al*, 1992; Phale *et al*, 2001) and characterization of its channel properties (Benz *et al*, 1978; Schindler and Rosenbusch, 1981; Benz, 1988; Basle *et al*, 2004) have resulted in extensive investigations of the structure determinants of ion and solute flow through the *E. coli* OmpF porin channel using theoretical and computational analysis (Karshikoff *et al*, 1994; Tieleman and Berendsen, 1998; Im and Roux, 2002; Roux *et al*, 2004; Varma and Jakobsson, 2004; Varma *et al*, 2006).

The OM vitamin B₁₂ receptor, BtuB, has previously been identified by genetic analysis as a primary receptor for the E colicins (Benedetti *et al*, 1989) and phage BF23 (Di Masi *et al*, 1973). In a purified state, BtuB was found to bind colicin E3 tightly, $K_d < 10^{-9}$ M (Taylor *et al*, 1998). Crystal structures of a complex of BtuB and the receptor (R) binding domain of colicin E3 (Kurisu *et al*, 2003) or E2 (Sharma *et al*, 2007) showed that the 100-Å-long coiled-coil R-domain was bound to BtuB in an oblique manner that did not displace the BtuB plug domain. The structure, and the absence of ionic current induced by colicin added to BtuB embedded in planar membrane bilayers (Zakharov *et al*, 2004), did not provide any suggestion that the colicin or one of its domains could be inserted into the cell through BtuB. A second OM protein, OmpF or OmpC, is known to be required for cytotoxicity of nuclease colicins (Mock and Pugsley, 1982; Sharma *et al*, 2007). It has been inferred from the crystal structure of the complex of BtuB and the R-domains of colicin E2 (Sharma *et al*, 2007) or E3 (Kurisu *et al*, 2003), colicin occlusion of the OmpF ion channel (Zakharov *et al*, 2004, 2006), and isolation of a colicin E9–BtuB complex with partial occupancy of

*Corresponding author. Department of Biological Sciences, Purdue University, Lilly Hall of Life Sciences, West Lafayette, Indiana 47907-1392, USA. Tel.: +1 765 494 4956; Fax: +1 765 496 1189; E-mail: waclab@purdue.edu

³Present address: Institute for Protein Research, Osaka University, Osaka, Japan

Received: 15 April 2008; accepted: 24 June 2008; published online: 17 July 2008

OmpF (Housden *et al*, 2005) that OmpF provides the channel across the OM for translocation of nuclease E colicins. This channel occlusion function of the colicin is similar to occlusion by the anthrax toxin 'LF' subunit of channels formed in planar bilayer membranes by the protective antigen component of anthrax toxin (Zhang *et al*, 2004). It has been proposed that the oblique orientation formed by the 100-Å-long colicin R-domain with the membrane plane provides a bridge or 'fishing pole' from BtuB to OmpF or OmpC on the extracellular side of the OM. This 'bridge' would allow formation of a translocon for cellular import of the colicin catalytic domain through the OM (Kurisu *et al*, 2003; Zakharov *et al*, 2004, 2006; Housden *et al*, 2005; Sharma *et al*, 2007).

OmpF can also serve as the sole OM-binding protein for import of colicin N, which apparently uses it as both receptor and translocator (Bourdineaud *et al*, 1990; El-Kouhen *et al*, 1993; Jeanteur *et al*, 1994; Baboolal *et al*, 2008), as also proposed for the *cir* OM receptor of colicin Ia (Buchanan *et al*, 2007).

In the present study, the hypothesis that unfolded domains of colicin E3 can use OmpF as a translocator was examined through biochemical and crystallographic analysis of interactions of OmpF with the unfolded N-terminal domain (T83) of colicin E3. Optimum crystallization conditions were found in the presence of 1 M Mg^{2+} , which yielded a 1.6 Å structure of OmpF without inserted T83. However, in the absence of Mg^{2+} , a 3.0 Å OmpF structure could be obtained from OmpF with an incremental electron density, which was attributed to a segment of T83 that spans most of the pore.

Results

As described in the original studies on the OmpF structure, each monomer in the *E. coli* OmpF trimer consists of a β -barrel made up of 16 amphipathic β -strands (Cowan *et al*, 1992), which defines an aqueous channel that spans the OM, with eight extended loops L1–L8 on the extracellular side of the barrel monomer, and eight tight turns on the periplasmic side (Figures 1A and B). The staves of the barrel surround a

water-filled pore with a narrow elliptically shaped (7×11 Å) selectivity filter having a solvent accessible area of $30\text{--}40$ Å² (Cowan *et al*, 1992; Varma *et al*, 2006). This area of limiting access in the channel is mostly defined by loop L3 (Arg100–G134), which connects β -strands 5 and 6 and is bent towards the inside of the barrel, positioned asymmetrically towards the extracellular side from the mid-plane of the membrane, as seen in a view parallel to the plane of the membrane (Figure 1B; ribbon structure in blue; loop 3 in purple). The acidic residues, Asp113 and Glu117 in this loop, and the side chains of the basic residues, Lys16, Arg42, Arg82 and Arg132, across the restriction zone on the inside of the barrel define a size-selective molecular filter that constricts the channel (Figures 1B and 2A).

High-resolution (1.6 Å) crystal structure of OmpF

To examine the function of OmpF in colicin import, co-crystallization trials were initiated with OmpF and the glycine-rich N-terminal domain of colicin E3 (T83), which is disordered in the crystal structure of the intact colicin (Soelaiman *et al*, 2001). Crystals formed in the presence of 1 M $MgCl_2$ resulted in an OmpF structure with a resolution substantially higher than that previously attained, although the colicin peptide was not seen. The crystal structure was determined to a resolution of 1.6 Å (Table I). The new structure (Figure 1B, barrel ribbons in green; loop 3 in orange) was not changed significantly relative to the 2.2 Å structure (Figure 1B, barrel in blue; loop 3 in purple) previously determined using a Tyr106Phe mutant of OmpF (Phale *et al*, 2001). The RMSD between the two structures is 0.26 Å.

The $F_o\text{--}F_c$ difference map in the 1.6 Å OmpF structure obtained from crystals formed in the presence of 1 M $MgCl_2$ shows, in a cross-section view parallel to the plane of the membrane, the presence of additional electron density arising from a $(H_2O)_6$ -coordinated Mg^{2+} ion (orange) near Asp113, Leu115 and Glu117 in the L3 loop of the selectivity filter (Figure 2A). Also shown is the positively charged array Arg42, Arg82, Arg132 and Lys16 on the opposite side of the limiting filter aperture, which implies the presence of a

Figure 1 (A) Ribbon diagram description of OmpF trimer determined at 1.6 Å resolution. The structure is tilted forward 20° from a vertical position of the trimer threefold symmetry axis; monomers are coloured in yellow, blue and green; two molecules of the detergent, octyl-tetraoxyethylene, are bound per monomer. (B) Superimposed ribbon diagrams of the structure of the monomer of the OmpF porin with the 1.6 Å resolution of wild-type porin obtained in this study and 2.2 Å resolution structure of the Tyr106Phe mutant (Phale *et al*, 2001), viewed parallel to the plane of the membrane. The r.m.s.d. between the backbone atoms of the two structures is 0.26 Å. The 1.6 and 2.2 Å structures are coloured green and blue (barrel), and orange and purple (loop 3), respectively.

Figure 2 (A) F_0-F_c difference map in the 1.6 Å OmpF structure showing the electron density of a hexa- H_2O -coordinated Mg^{2+} atom (orange) near Asp113, Leu115 and Glu117 in the L3 loop of the selectivity filter. Surrounding OmpF barrel region, green; loop 3, purple. Also shown are Arg42, Arg82, Arg132, Lys16, Lys80 and Glu62 on the opposite side of the limiting filter aperture. Other charged and Tyr residues that define the boundaries of the limiting filter aperture are Lys312, Glu117, Tyr102 and Tyr106. The cross-sectional dimensions of the limiting aperture are 7.8 Å (Arg82NH–Asp113OD2)—8.8 Å (Lys16NZ–Leu115O) $\times$ 14.4 Å (Tyr310OH–Tyr106OH, or Tyr310OH–Tyr102OH). (B) Hydrogen-bonding pattern of $(H_2O)_6-Mg^{2+}$ to Asp113OD1 and to the backbone carbonyls of Leu115 and Glu117. Residues removed for purposes of clarity in Figure 2B are 25–34 in the loop 1, 161–170 in loop 4, 197–209 in loop 5, 237–251 in loop 6 and 318–329 in loop 8.

Table 1 Intensity data and refinement statistics: OmpF and OmpF/T83

OmpF structure	OmpF (in Mg^{2+})	OmpF/T83 (no Mg^{2+})
<i>Crystal</i>		
Space group	$P3_21$	$P6_3$
Cell constants		
a, b (Å)	116.9	116.9
c (Å)	51.3	114.3
<i>Data collection</i>		
Resolution (Å)	1.59 (1.65–1.59)	3.0 (3.00–3.11)
Measured reflections	450 302 (25 201)	147 824 (14 623)
Unique reflections	53 879 (5143)	17 885 (1765)
Redundancy	8.4 (4.9)	8.3 (8.3)
I/σ (I)	35.1 (3.0)	24.4 (8.6)
Completeness (%)	99.5 (96.0)	99.9 (100.0)
R_{merge}^a	0.079 (0.402)	0.076 (0.261)
<i>Refinement</i>		
R^b	0.182	0.266
R_{free}^c	0.218	0.294
r.m.s. deviation from ideal		
Bond lengths (Å)	0.01	0.01
Bond angles (deg)	1.2	0.98

Values in parentheses apply to the highest resolution shell.

^a $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where I_i is the intensity of the measured reflection.

^b $R = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_c and F_o are the calculated and observed structure factors, respectively.

^c R_{free} is calculated for a randomly chosen 5.0% of reflections omitted from refinement.

transverse electric field normal to the channel axis (Karshikoff *et al*, 1994; Tieleman and Berendsen, 1998; Im and Roux, 2002). Other residues that define the boundaries of the filter are Lys312, Glu117, Tyr102 and Tyr106. The cross section of the limiting aperture of the selectivity filter is 7.8 Å (Arg82NH–Asp113OD2)—8.8 Å (Lys16NZ–Leu115O) $\times$ 14.4 Å (Tyr310OH–Tyr106OH, or Tyr310OH–Tyr102OH).

On the basis of 1.6 Å resolution of the structure and the associated R factors, the uncertainty in these distances

is ± 0.2 Å. The solvent-accessible cross-sectional area subtended by these distances is elliptically shaped, with axes of 7 $\times$ 13 Å, measured edge to edge, slightly larger than the 7 $\times$ 11 Å cross section derived from the original OmpF structure (Cowan *et al*, 1992). The hydrogen-bonding pattern of the $(H_2O)_6-Mg^{2+}$ to Asp113OD1 and to the backbone carbonyls of Leu115 and Glu117 is shown, along with the approximate distances spanned by these bonds (Figure 2B). Two additional water molecules (small red spheres) are ligands to two of six water molecules bound to magnesium. One of the six water molecules bound to magnesium has two hydrogen bonds, D113 and one of the two additional water molecules. Thus, four of the six water ligands of the magnesium are associated with demonstrable ligands. Site-directed mutations of the two carboxylates and three Arg residues in the constriction zone, and of adjacent residues, imply a function of the L3 loop in ionic conductance as well as colicin interactions (Jeanteur *et al*, 1994; Phale *et al*, 2001; Bredin *et al*, 2003).

It had been inferred that changes in the size of the pore can arise from fluctuations in position of the residues Pro116–Glu117–Phe118– α Glu119–Gly120 in the L3 loop, which were predicted to form the most flexible region in the narrow selectivity filter region of the pore (Im and Roux, 2002), with Pro116 displaying the largest deviation (± 4 Å). The temperature (B) factors calculated from the present 1.6 Å structure do not indicate a unique effect on any individual residue compared with the 2.2 Å (pdb: 1HXX) structure (Phale *et al*, 2001). The average temperature (B) factor of the residues in the Pro116–Gly120 segment (19.6 Å²) is slightly smaller than the average for the whole molecule (24.5 Å²). Although the Mg^{2+} ion binds in the L3 loop, there is no residue in this loop that shows an unusually large decrease in B value. The B factors of the 1.6 Å versus the 2.2 Å OmpF structures for other parts of the structure are compared: (a) average for whole molecule, 24.5 versus 32.8 Å²; (b) extracellular loops, 27.6 versus 37.5 Å²; (c) extracellular loops 1 and 7, with the largest B factors, 30.8

versus $47.3 \text{ \AA}^2$ and 42.8 versus $65.2 \text{ \AA}^2$, respectively; (d) barrel staves, 22.0 versus $28.7 \text{ \AA}^2$. Thus, a global tightening of the structure associated with the higher resolution structure cannot be attributed to any single residue or subset of residues in the L3 loop or elsewhere.

Effect of bound Mg^{2+} on channel activity. The $(\text{H}_2\text{O})_6$ -coordinated Mg^{2+} that is hydrogen bonded to the three residues in the L3 loop protrudes into the solvent-accessible space of the filter (Figures 2A and B). It partly occludes this channel aperture, resulting in decreased ion flow. Indeed, the single channel current of OmpF in 0.5 – 1.0 M NaCl was approximately 10% smaller in the presence of a concentration (33 mM) of MgCl_2 (data not shown) that is too small to be responsible for the decrease of conductance.

Ordered water and structure of OmpF. The $1.6 \text{ \AA}$ structure is otherwise similar in atomic detail except for the number of ordered H_2O molecules resolved within the channel, which increased from the 103 determined in the $2.2 \text{ \AA}$ structure (Phale *et al*, 2001) to 214 in the $1.6 \text{ \AA}$ structure, 108 in the barrel, including 41 in the selectivity filter, and 86 and 20, respectively, in the extracellular and periplasmic domains (Supplementary Figure 1). This number of ordered waters within the OmpF channel may be a common property of 16-stranded porins such as OmpF, as the 1.80 and $1.96 \text{ \AA}$ porin structures found in the photosynthetic bacteria, *Rhodobacter capsulatus* (Weiss and Schulz, 1992) and *Rhodospseudomonas blastica* (Kreusch and Schulz, 1994) contain 173 and 154 internal H_2O , respectively. The *E. coli* OmpC porin at a resolution of $2.0 \text{ \AA}$ contains 111 immobilized internal waters (Basle *et al*, 2006).

OmpF forms a complex with the T83 peptide of colicin E3

Occlusion of OmpF channel conductance by T83. When added to the *trans*-side of a planar bilayer membrane, the side opposite to that of OmpF addition, colicin E3 occluded OmpF channels formed in planar bilayer membranes (Zakharov *et al*, 2004). The T83 construct that mimics the N-terminal 83 residues of colicin E3, added from the *trans*-side of a planar bilayer membrane, also occludes OmpF channels (Figures 3A and B). Occlusion of OmpF channels by colicin E3 requires the presence of a *cis*-negative voltage. Application of a *cis*-positive voltage reverses occlusion (Zakharov *et al*, 2004). In contrast to full-length colicin E3 (Kurisu *et al*, 2003; Zakharov *et al*, 2004), occlusion of OmpF channels by T83 does not require a *cis*-negative potential (Figure 3B), as T83 is able to occlude OmpF without the application of voltage (data not shown). Even with the application of a *cis*-positive voltage, the occlusion was not reversed completely. However, as was the case with colicin E3, T83 does not occlude OmpF channels when added to the *cis*-side. Deletion of the T83 segment from intact colicin prevented occlusion, as did the mutational changes Asp5Ala/Arg7Ala (Zakharov *et al*, 2004), implying that an interaction site with OmpF is located in the N-terminal segment of the colicin E3 T-domain.

It is important to note that PEG 6000, used in the co-crystallization of OmpF and T83, did not occlude OmpF channels, even when added at a molar concentration

Figure 3 Occlusion of OmpF channels by T83. Planar bilayer membranes were formed of DOPC and DOPE (1:1, w/w) dissolved in *n*-decane. OmpF in 0.7% octyl-POE was added to the *cis*-side of the membrane (ca. 10 pg/ml). The sign of the trans-membrane potential is shown for the *cis*-side of the membrane. Trans-membrane current (grey traces) was (A) $+156 \text{ pA}$ ($+50 \text{ mV}$) and (B) -192 pA (-50 mV) implying opening of 12 OmpF channels (four trimers). After recording OmpF channels, T83 ($0.2 \text{ \mu g/ml}$) was added to the *trans*-compartment. Recording started at -50 mV revealed an occluded state of all channels with sporadic opening of 1 or 2 channels (lower panel). (A) Switching the potential to $+50 \text{ mV}$ partially restored the open state of the OmpF channels.

10^3 – 10^4 times that of T83 (data not shown). However, subsequent addition of T83 in the presence of the PEG caused occlusion of OmpF channels.

OmpF binds T83 in the absence of Mg^{2+} ; size exclusion chromatography. T83 and OmpF, mixed at an equimolar ratio (per monomer), added in the absence of Mg^{2+} , were separated by size exclusion chromatography to remove unbound T83. A significant amount of T83 was co-eluted from the column together with trimeric OmpF (peak, 11.0 ml ; Figure 4A). The presence of the T83 band in this peak (Figure 4B), detected by SDS-PAGE, implied formation of a tight complex between trimeric OmpF and T83. However, T83 alone is eluted at the position expected for an 8.3 kDa monomer and did not show the presence of oligomeric T83 (Figure 4A, dashed curve). Comparison of intensities of a stained T83 band in the complex and bands with a known quantity of purified T83 (Figure 4C) allowed an estimate of the molar ratio of trimeric OmpF to T83 in this complex. Assuming 0.15 – $0.20 \text{ \mu g}$ of T83 in band 1, the molar ratio of T83 per OmpF trimer was 1–2.

Immunoprecipitation. According to a translocon model of colicin E3 import (Kurisu *et al*, 2003; Housden *et al*, 2005; Zakharov *et al*, 2006; Sharma *et al*, 2007), BtuB-bound colicin E3 locates, and binds to, OmpF porin using its N-terminal-flexible ‘fishing line’ consisting of T83. In the present study, this step of import was confirmed by immunoblot detection

Figure 4 Complex of OmpF and colicin E3 N-terminal T83 peptide detected by size-exclusion chromatography. **(A)** OmpF was passed through a Superdex 200 (10/300) column (thin line) in 0.7% octyl-POE buffer as described in Materials and methods. OmpF trimer (fractions 10–12.5 ml from previous run), 9.2 nmol, was mixed with T83, 25 nmol, and was passed through the same column (bold line). The absence of T83 oligomers, which could mimic formation of the OmpF/T83 complex, was confirmed by the elution of T83 alone, 130 nmol, through the same column (dashed line). All data are normalized to a common maximum ordinate. **(B)** SDS-PAGE of proteins eluted from Superdex 200 column. (1) Protein standards; (2) OmpF trimer (peak 11.1 ml from the first run); (3) OmpF-T83 complex (second run, peak 11.0 ml); (4) OmpF monomer (second run, peak 14.6 ml); (5) T83 (peak 17.8 ml). SDS-PAGE was run in 15% polyacrylamide. **(C)** Estimate of T83 content complexed to OmpF trimer. SDS-PAGE: 1, OmpF/T83 from elution peak at 11.0 ml; 2, 3, 4 and 5, T83, 0.125, 0.25, 0.5 and 1.0 μ g, respectively. The content of the OmpF trimer in the elution peak at 11.0 ml (5.3 μ M) was determined spectrophotometrically, using a molar extinction coefficient (ϵ_M) at 280 nm of 1.63×10^5 , and neglecting the contribution of T83 ($\epsilon_M = 1.65 \times 10^4$) to the absorbance of the OmpF trimer. Assuming that the intensity of the Coomassie stain of T83 bands in samples 1 and 3 is similar, a 10 μ l aliquot applied to SDS-PAGE contained $\sim 0.25 \mu$ g T83, implying that each OmpF trimer in the peak at 11.0 ml bound 1–2 molecules of the T83 peptide.

of the complex between colicin E3, BtuB and OmpF (Figure 5). *E. coli* membranes were incubated with colicin E3, and then cross-linked with 1% formaldehyde. After protein extraction with β -D-octyl-glucoside (OG), complexes containing colicin E3 were immunoprecipitated using anti-C-domain antibodies conjugated with CNBr-activated Sepharose beads, subjected to SDS-PAGE, and transferred to a blot membrane. Immunoblot detection using antibodies against BtuB, OmpF and colicin E3 revealed colicin E3 in a complex with BtuB and OmpF (Figure 5). This result confirms a previous report of formation of a ternary complex between colicin E9 and BtuB, together with OmpF at a 30% stoichiometry, detected using a Ni-affinity column (Housden *et al*, 2005).

3.0 Å structure of OmpF with inserted segment of T83; F_o minus F_c map of OmpF and inserted peptide. Co-crystallization of T83 with OmpF in the absence of Mg^{2+} resulted in crystals that diffracted to 3.0 Å in a different space group

Figure 5 Immunodetection of the complex between BtuB, OmpF and colicin E3. *E. coli* membranes were incubated with colicin E3, 0.26 μ M, and then cross-linked with 1% formaldehyde. Proteins were extracted with 3% OG. Complexes containing colicin E3 were immunoprecipitated using anti-C-domain antibodies conjugated with CNBr-activated Sepharose beads, subjected to SDS-PAGE, transferred to a PVDF membrane and immunodetected using antibodies against BtuB, OmpF and colicin E3. Immunoblot samples: 1, protein standards; 2, 4 and 6, isolated OmpF, BtuB and colicin E3, respectively; 3, 5 and 7, membrane protein extract. Samples 2 and 3, 4 and 5, 6 and 7 were incubated with anti-OmpF, anti-BtuB and anti-colicin E3 antibodies, respectively.

(P6₃ versus P321). A difference Fourier map from the OmpF-T83 data derived from the OmpF model, contoured at 3.0 σ , shows additional electron density (in magenta) in the selectivity filter that extends from the aperture in loop 3 on the extracellular side (stereo view, Figure 6A) of the selectivity filter almost to the periplasmic side of the enclosed barrel. The continuous additional electron density resembles a peptide containing at least seven residues that extends over 18 Å. It is modelled with the NH₂-terminus pointing to the periplasmic side (bottom, Figure 6A). The refinement parameters for this model are $R = 0.266$ and $R_{\text{free}} = 0.294$ (Table I). Additional perspectives of the OmpF structure with the superimposed incremental electron densities obtained in the absence and presence of 1 M Mg^{2+} during crystallization are shown in a view along the OmpF axis (Figure 6B; peptide in stick model), and in a view emphasizing the proximity of the putative peptide to loop 3 of OmpF and the Mg^{2+} -binding site (Figure 6C). The inserted peptide was modelled as a polypeptide backbone that contains mostly disordered side chains. A putative side chain seen near the middle of the peptide, which may be partly ordered by the proximal loop 3, resembles that of an Asp, Asn or Glu residue, which are present in T83. The N- and C-terminal hepta-peptide sequences of the T83 constructs are (M1)-S-G-G-D-G-R-G8 and T77-G-G-N-L-S-A83-LE-(His)₈. In planar bilayer experiments, the T83 peptide must enter the porin channel via the N-terminus because colicin with an N-terminal His tag does not occlude (Zakharov *et al*, 2006) and the T83 construct contains a C-terminal (His)₈-tag. However, under the *in vitro* conditions with isolated OmpF in detergent, and at the present level of resolution, it is not known whether the peptide enters OmpF through its extracellular or periplasmic sides. An alternative possibility for the extra density is that it arises from a string of the PEG 6000 precipitant that was used in the crystallization. This alternative was considered unlikely, as discussed below.

Discussion

The higher-resolution OmpF structure

Two obvious reasons for the improvement in the resolution of the *E. coli* OmpF structure relative to the best resolution

Figure 6 Interaction of an incremental electron density (magenta) associated with a peptide segment of T83 bound in the OmpF pore. (A) Stereo view of OmpF and the F_0-F_c map of the additional electron density attributed to a segment of the T83 peptide. L3 loop is shown in yellow with its helical sequences in red. The proximity of the peptide density to the binding site of a Mg^{2+} ion is shown. The additional density attributed to the T83 peptide consists of at least seven residues and extends over a distance of at least 18 Å. Top, extracellular, and bottom, periplasmic side of OmpF. (B) View along axis of selectivity filter (cf., Figure 2B) showing superimposed difference densities of the peptide and the bound Mg^{2+} ion seen in the absence of T83. (C) Region of interaction of T83 peptide segment (electron density map in blue), loop 3 of OmpF and bound Mg^{2+} ion, shown in enlarged format.

previously attained (Cowan *et al*, 1992; Phale *et al*, 2001) are as follows: (i) it results from the presence of the $(H_2O)_6-Mg^{2+}$ bound between the two carboxylate residues in loop 3 of the selectivity filter (Figures 2A and B); (ii) a different and, perhaps most importantly, much more rapid procedure was used for the isolation of OmpF. Although it could be inferred that the improved resolution is a consequence of a tightening of the structure in the presence of the bound magnesium ion, the purification procedure used in this study is simpler and faster than that used previously for the preparation of OmpF (Garavito and Rosenbusch, 1986) and also involves the use of a different detergent (OG) for extraction (see Materials and methods). One of the purposes of the extended procedure used previously was to remove lipopolysaccharide (LPS). LPS was not seen in the 1.6 Å structure. It should be noted that the presence of the Mg^{2+} in the structure, which was a consequence of incubation with very high concentrations of $MgCl_2$, is not considered to be physiologically significant beyond serving as an indicator of the cation selectivity of the OmpF ion channel. In this study, the Mg^{2+} was utilized initially because it was shown to enhance crystal formation in initial crystallization screening experiments. However, in the final

analysis, the overlap of the density of the Mg^{2+} and the incremental density obtained in the absence of Mg^{2+} and the presence of T83 yielded information about the location of the extra density obtained with T83.

The identity of the incremental electron density in the presence of T83

Given the possible ambiguity in the electron density at 3.0 Å resolution, there are several experimental points that support the inference that the increment of the density relative to that of OmpF alone belongs to an embedded segment of the T83 peptide. (i) In the experiment intended to separate an OmpF–T83 complex by size exclusion chromatography, OmpF binds T83 in the absence but not the presence of Mg^{2+} , consistent with the inability to crystallize OmpF/T83 under these conditions. (ii) Comparison of the electron density obtained in the presence of Mg^{2+} and in the presence of T83 without Mg^{2+} provides the explanation for the Mg^{2+} effect (Figures 6B and C). The model of the T83 density, obtained in the absence of Mg^{2+} , shows that its incremental electron density overlaps that of the Mg^{2+} -binding site. T83 competes with Mg^{2+} for a binding site near Asp113. Thus, the additional

density obtained in the presence of T83 has a cationic character, which is not a property of PEG 6000. (iii) The model also indicates that the putative peptide is adjacent to the L3 loop (Figure 6C), with a distance of closest approach of 3.0–3.5 Å to Asp113, suggesting the possibility of an H-bond between a side chain and the carboxylate of Asp113. (iv) Addition of PEG 6000 at a molar concentration 10^3 – 10^4 times that of T83 used to demonstrate channel occlusion in Figure 3B did not show any occlusion of the OmpF channel conductance, and does not prevent occlusion by T83. (v) Although most of the electron density that would be associated with amino-acid side chains in the peptide is not seen, presumably because of the glycine-rich nature of T83, local disorder, and lack of resolution at 3.0 Å, a branched density is seen near the middle of the peptide. This density extends to the β -barrel wall underneath loop 3 (Figure 6A). Such a density cannot be associated with a PEG molecule.

Protein import; translocons in protein trafficking

The OM colicin import system is one of numerous examples of a *trans*-membrane protein trafficking system. However, it is the only one in which a part of the transportable protein has been seen in the crystal structure. Membrane proteins that function in protein import/export/secretion are ubiquitous (Osborne *et al*, 2005; Wickner and Schekman, 2005). Multi-protein arrays are known to be required for protein translocation across membranes in secretion (Lee and Schneewind, 2001) and in protein import into organelles (Pfanner *et al*, 2004; Baker *et al*, 2007; Neupert and Herrmann, 2007; Bolender *et al*, 2008).

The polypeptide composition of the protein import machinery has been analysed extensively in organelles, especially mitochondria (Pfanner *et al*, 2004; Baker *et al*, 2007; Neupert and Herrmann, 2007; Bolender *et al*, 2008), where a total of at least 32 intramembrane subunits have been identified that direct traffic across the outer and inner membranes (IMs). However, even though the subunit components of the mitochondrial protein transport have been extensively documented, a structure of the transporter in complex with the peptide to be transported is yet to be reported (Baker *et al*, 2007).

A crystal structure (3.2 Å) for the heterotrimeric Sec61 putative protein conducting channel from *Methanococcus jannaschii* (Van den Berg *et al*, 2004) shows an internal funnel-like structure that is suggestive of a polypeptide translocation channel. However, thus far there is no crystal structure of a Sec61 complex that shows a polypeptide inserted into a Sec61, although translocation of a plant viral protein through a Sec61 system has been implied by photo-crosslinking (Sauri *et al*, 2007).

Studies on auto-transporter virulence factors, for which crystal structures are available from *E. coli*, *Hemophilus influenzae* and *Neisseria meningitidis* (Oomen *et al*, 2004; Meng *et al*, 2006; Barnard *et al*, 2007) have provided a structural precedent for protein translocation across the OM of Gram-negative bacteria. Although the virulence domain is in a folded conformation as seen in the auto-translocator structure, it was predicted that the colicin polypeptide must be unfolded to pass through the OmpF porin (Kurusu *et al*, 2003) because of the small limiting dimensions of the OmpF pore seen in the original crystal structure (Cowan *et al*, 1992).

Occlusion of OmpF channels by the disordered 83 residue N-terminal segment of colicin E3 (Figure 3), binding of T83 to OmpF (Figure 4) and identification of a complex between BtuB, OmpF and colicin E3 (Figure 5) provided a precedent for the crystallization trials that resulted in the 1.6 Å structure of OmpF alone under conditions of high ionic (Mg^{2+}) strength (Figures 1 and 2) and the 3.0 Å structure of OmpF with inserted T83 (Figures 6A–C). The failure of colicin E3 or its 83 residue N-terminal disordered polypeptide to occlude OmpF channels when the two N-terminal residues, Asp5 and Arg 7, were changed to neutral residues was attributed to interaction of the disordered N-terminal domain of colicin E3 with charged residues inside the OmpF pore (Zakharov *et al*, 2004).

The colicin translocon

T83 occlusion of OmpF channels, binding of T83 to OmpF *in vitro*, complex formation between BtuB, colicin E3 and OmpF and the crystal structure showing T83 inserted into OmpF provide extensive experimental support for an OM translocon for colicin import. An analogous model for import of the A-type colicin E1 would utilize the TolC *trans*-envelope protein instead of OmpF (Zakharov *et al*, 2004). Of course, many other functions associated with this translocon model are presently not understood. TolB, which is connected to the Tol network embedded in the cytoplasmic membrane, is believed to bind the N-terminally located TolB box of the colicin T-domain in order to provide the energy to pull the T-domain through the OmpF pore (Loftus *et al*, 2006; Bonsor *et al*, 2007). The net function of the T-domain is to prime the translocon for import of the C-domain that contains the activity, rRNase in the case of colicin E3. The mechanism of coupling of translocation of T- and C-domains is not understood at present, although it is known that occlusion of the OmpF pore by the C-domain requires the latter to be unfolded (Zakharov *et al*, 2006). It can also be noted that the necessity of coupling of the translocation of T- and C-domains provides a *raison d'être* for a multimeric (at least dimeric) OmpF.

Alternative use of OmpF by colicin N

It has been proposed that colicin N uses the interface between OmpF and the OM lipid/LPS for translocation through the OM, on the basis of an electron microscope difference analysis at 25 Å resolution of two-dimensional crystals of OmpF in the presence and absence of colicin N. A small change in electron density on the OmpF surface was attributed to bound colicin N (Baboolal *et al*, 2008). Although the identification of the extra density with colicin N may be considered to be somewhat uncertain at 25 Å resolution, the site identified with interaction of colicin N on OmpF could be an initial binding or contact site that is also used by other A-type colicins such as E3 that subsequently insert into an OmpF pore.

Materials and methods

Bacterial strains, growth conditions; peptide preparation

OmpF was purified from *E. coli* strain MH225 (pPR272), which was kindly provided by Professor P Loll and JB Kaplan. Cells were grown in LB broth containing kanamycin at 37 °C to an optical density, $OD_{600} \approx 1.5$ – 2.0 . The T83 N-terminal peptide of colicin E3 was prepared as demonstrated by Zakharov *et al* (2006).

Isolation of OmpF

IM solubilization. Except as noted, isolation of OMs was carried out as shown by Taylor *et al* (1998), which is on the basis of IM solubilization by Triton X-100.

OmpF purification. OMs were extracted with 3% OG (Anatrace) in 20 mM Tris, pH 8.0, 1 mM EDTA, incubated at room temperature for 2 h with stirring, and centrifuged for 40 min at 120 000 g, and the sediment was extracted again with 3% OG. Combined supernatants were loaded onto the FPLC Q column (HiPrep FF 16/10, Amersham) equilibrated with 20 mM Tris, pH 8.0, and 0.8% *N*-octyl-oligoxyethylene (octyl-POE, Alexis). Unbound proteins were washed out, and bound proteins were eluted with 20 mM Tris, pH 8.0, 0.8% octyl-POE and a 0–0.8 M gradient of LiCl. Eluted fractions were analysed with SDS–PAGE (12.5% acrylamide). For crystallization, fractions containing OmpF from the first OmpF peak of the Q column were concentrated in a 50 kDa cut-off Centrprep-50 (Millipore), with the buffer exchanged to 20 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 0.8% octyl-POE during concentration or on a Superdex 200 HR 10/30 gel filtration column (Amersham).

Crystallization

1.6 Å structure of OmpF. Crystals were formed by hanging drop vapour diffusion using VDX plates (Hampton Research) with the mother liquor solution, 100 mM Tris, pH 7.5, 14% PEG 2000, 1 M MgCl₂, mixed in a 1:1 ratio with the protein solution, 20 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 0.8% octyl-POE; OmpF concentration, 5 mg/ml. The N-terminal 83 residue segment, T83, of colicin E3 was present in the crystallization solution at a 1:1 molar ratio with OmpF, but was not found in the final electron density.

Co-crystallization of OmpF and T83; 3.0 Å structure of OmpF and T83. The mother liquor solution, 100 mM MES, pH 6.5, 15% PEG 6000, 5% MPD, was mixed in a 1:1 ratio with the protein solution, 20 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 0.8% octyl-POE. Molar ratio of T83:OmpF = 1:1.5.

Cryo-freezing

OmpF. Crystals were stabilized in 100 mM Tris, pH 7.5, 21% PEG 2000, 1.2 M MgCl₂, 0.8% octyl-POE, 50 mM NaCl for 20–30 min, then incubated for 5 min in solutions containing 5–25% glycerol, incremented by 5% in each step. After the final incubation in 25% glycerol solution, crystals were frozen in liquid N₂.

OmpF and T83. Crystals were stabilized in 100 mM MES, pH 6.5, 18% PEG 6000, 6% MPD, 0.8% octyl-POE, 50 mM NaCl for 20–30 min, then incubated in cryoprotectant solutions containing 6–35% MPD for 2 min, incremented by 4% in each step.

Collection and analysis of diffraction data

X-ray diffraction data were collected at beamline SBC 19-ID at the Advanced Photon Source (Argonne National Lab). Processing of diffraction data was carried out with HKL2000 (Otwinowski and Minor, 1997). The structure was determined by rigid body refinement with the program REFMAC5 (Murshudov *et al*, 1997) in CCP4 (Bailey, 1994) using the original *E. coli* OmpF structure (pdb: 2OMF) as an initial model. The structure refinement and rebuilt models used the programs REFMAC5 and 'O' (Jones *et al*, 1991). A summary of the crystallographic data and refinement statistics is given in Table I. The programs Molscript (Kraulis, 1991), Povscript (Fenn *et al*, 2003) and Raster3D (Merritt and Bacon, 1997) were used for the molecular graphic presentation. The minimum

radius of the pore, determined by the program HOLE (Smart *et al*, 1993), is 3.4 Å.

OmpF channel measurements in planar bilayers

Planar bilayer membranes were formed on a 0.2 mm diameter aperture in a partition that separates two 4 ml compartments, using a 1:1 (mol:mol) mixture of the two lipids (Avanti), dioleoyl-phosphatidylcholine and dioleoyl-phosphatidylethanolamine (10 mg/ml) in *n*-decane, applied by a brush technique (Mueller *et al*, 1962). The aqueous solution in both compartments consisted of 5 mM KPi, pH 7.0, 0.1 M KCl. OmpF, 0.1–2 µl of 0.1–10 ng/ml solution in 1% octyl-POE, was added to the *cis*-compartment and the solution was stirred until channels appeared. The *trans*-membrane current was measured in the voltage-clamp mode with Ag/AgCl electrodes, using a BC-525C amplifier (Warner Instruments, Hamden, CT, USA). The *trans*-membrane potential was applied to the electrode on the *cis*-side of the membrane.

Size-exclusion chromatography

Size-exclusion chromatography of OmpF/T83 mixture was conducted on the Superdex 200 column (10/300) in 0.7% octyl-POE, 20 mM HEPES, pH 7.8, 20 mM NaCl. A Centricon 50 filter was used to concentrate OmpF alone or in a complex with T83. The content of OmpF trimer (4.2 µM) was determined spectrophotometrically, using an ϵ_{mM} (280 nm) = 163, neglecting the contribution of T83 (ϵ_{mM} = 16.5). Assuming that the intensity of the T83 bands in samples 1 and 3 is similar (Figure 4C), it was inferred that the OmpF/T83 complex contains approximately 0.2 µg of T83; stoichiometry, 1–2 mol T83 per a mol of OmpF trimer.

SDS–PAGE

SDS–PAGE was carried out according to Laemmli (1970) using 12% acrylamide gels.

Protein cross-linking and immunodetection

Escherichia coli (BL21) cells were disrupted by a French press and membranes were sedimented and resuspended in 0.1 M NaPi, pH 6.8. Before cross-linking, membranes were incubated with colicin E3, 0.26 µM for 10 min, and then treated with 1% formaldehyde for 1 h at room temperature. Proteins from membranes were extracted by 3% OG, and eluted through CNBr-activated Sepharose beads with conjugated antibodies to the C-domain of colicin E3. Adsorbed proteins were analysed by SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and immunochemically detected using antibodies against BtuB, OmpF and colicin E3.

PDB deposition

Structures deposited in Protein Data Bank: 1.6 Å OmpF structure (pdb: 2ZFG); 3.0 Å structure of OmpF with inserted segment of N-terminal peptide, T83, of colicin E3 (pdb: 2ZLD).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We gratefully acknowledge helpful discussions with D Baniulis and A Finkelstein. These studies were supported by NIH Grant GM18457, the Purdue Cancer Center, and the Henry Koffler Professorship (WAC). X-ray diffraction data were collected at beam line SBC19-ID at the Advanced Photon Source, Argonne National Laboratory (supported by DOE W31-109-ENG-389).

References

- Baboolal TG, Conroy MJ, Gill K, Ridley H, Visudtiphale V, Bullough PA, Lakey JH (2008) Colicin N binds to the periphery of its receptor and translocator, outer membrane protein F. *Structure* **16**: 371–379
- Bailey S (1994) The CCP4 suite—programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **50**: 760–763
- Baker MJ, Frazier AE, Gulbis JM, Ryan MT (2007) Mitochondrial protein-import machinery: correlating structure with function. *Trends Cell Biol* **17**: 456–464
- Barnard TJ, Dautin N, Lukacik P, Bernstein HD, Buchanan SK (2007) Autotransporter structure reveals intra-barrel cleavage followed by conformational changes. *Nat Struct Mol Biol* **14**: 1214–1220
- Basle A, Iyer R, Delcour AH (2004) Subconductance states in OmpF gating. *Biochim Biophys Acta* **1664**: 100–107
- Basle A, Rummel G, Storici P, Rosenbusch JP, Schirmer T (2006) Crystal structure of osmoporin OmpC from *E. coli* at 2.0 Å. *J Mol Biol* **362**: 933–942

- Benedetti H, Frenette M, Baty D, Llobes R, Geli V, Lazdunski C (1989) Comparison of the uptake systems for the entry of various BtuB group colicins into *Escherichia coli*. *J Gen Microbiol* **135**: 3413–3420
- Benz R (1988) Structure and function of porins from Gram-negative bacteria. *Annu Rev Microbiol* **42**: 359–393
- Benz R, Janko K, Boos W, Lauger P (1978) Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. *Biochim Biophys Acta* **511**: 305–319
- Bolender N, Sickmann A, Wagner R, Meisinger C, Pfanner N (2008) Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Rep* **9**: 42–49
- Bonsor DA, Grishkovskaya I, Dodson EJ, Kleanthous C (2007) Molecular mimicry enables competitive recruitment by a natively disordered protein. *J Am Chem Soc* **129**: 4800–4807
- Bourdineaud JP, Fierobe HP, Lazdunski C, Pages JM (1990) Involvement of OmpF during reception and translocation steps of colicin N entry. *Mol Microbiol* **4**: 1737–1743
- Bredin J, Simonet V, Iyer R, Delcour AH, Pages JM (2003) Colicins, spermine and cephalosporins: a competitive interaction with the OmpF eyelet. *Biochem J* **376**: 245–252
- Buchanan SK, Lukacik P, Grizot S, Ghirlando R, Ali MM, Barnard TJ, Jakes KS, Kienker PK, Esser L (2007) Structure of colicin I receptor bound to the R-domain of colicin Ia: implications for protein import. *EMBO J* **26**: 2594–2604
- Cascales E, Buchanan SK, Duche D, Kleanthous C, Llobes R, Postle K, Riley M, Slatin S, Cavard D (2007) Colicin biology. *Microbiol Mol Biol Rev* **71**: 158–229
- Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, Jansonius JN, Rosenbusch JP (1992) Crystal structures explain functional properties of two *E. coli* porins. *Nature* **358**: 727–733
- Di Masi DR, White JC, Schnaitman CA, Bradbeer C (1973) Transport of vitamin B12 in *Escherichia coli*: common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *J Bacteriol* **115**: 506–513
- El-Kouhen R, Fierobe HP, Scianimanico S, Steiert M, Pattus F, Pages JM (1993) Characterization of the receptor and translocator domains of colicin N. *Eur J Biochem* **214**: 635–639
- Fenn TD, Ringe D, Petsko GA (2003) POVScript+: a program for model and data visualization using persistence of vision ray-tracing. *J Appl Crystal* **36**: 944–947
- Garavito RM, Rosenbusch JP (1980) Three-dimensional crystals of an integral membrane protein: an initial x-ray analysis. *J Cell Biol* **86**: 327–329
- Garavito RM, Rosenbusch JP (1986) Isolation and crystallization of bacterial porin. *Meth Enzymol* **125**: 309–328
- Housden NG, Loftus SR, Moore GR, James R, Kleanthous C (2005) Cell entry mechanism of enzymatic bacterial colicins: porin recruitment and the thermodynamics of receptor binding. *Proc Natl Acad Sci USA* **102**: 13849–13854
- Im W, Roux B (2002) Ions and counterions in a biological channel: a molecular dynamics simulation of OmpF porin from *Escherichia coli* in an explicit membrane with 1 M KCl aqueous salt solution. *J Mol Biol* **319**: 1177–1197
- Jeanteur D, Schirmer T, Fourel D, Simonet V, Rummel G, Widmer C, Rosenbusch JP, Pattus F, Pages JM (1994) Structural and functional alterations of a colicin-resistant mutant of OmpF porin from *Escherichia coli*. *Proc Natl Acad Sci USA* **91**: 10675–10679
- Jones TA, Zou J-Y, Cowan SW, Kjeldgaard M (1991) Improved methods for the building of protein models in electron density maps and the location of errors in these models. *Acta Crystallograph A* **47**: 110–119
- Karshikoff A, Spassov V, Cowan SW, Ladenstein R, Schirmer T (1994) Electrostatic properties of two porin channels from *Escherichia coli*. *J Mol Biol* **240**: 372–384
- Kraulis PJ (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* **24**: 946–950
- Kreusch A, Schulz GE (1994) Refined structure of the porin from *Rhodospseudomonas blastica*. Comparison with the porin from *Rhodobacter capsulatus*. *J Mol Biol* **243**: 891–905
- Kurusu G, Zakharov SD, Zhalnina MV, Bano S, Eroukova VY, Rokitskaya TI, Antonenko YN, Wiener MC, Cramer WA (2003) The structure of BtuB with bound colicin E3 R-domain implies a translocon. *Nat Struct Biol* **10**: 948–954
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Lee VT, Schneewind O (2001) Protein secretion and the pathogenesis of bacterial infections. *Genes Dev* **15**: 1725–1752
- Loftus SR, Walker D, Mate MJ, Bonsor DA, James R, Moore GR, Kleanthous C (2006) Competitive recruitment of the periplasmic translocation portal TolB by a natively disordered domain of colicin E9. *Proc Natl Acad Sci USA* **103**: 12353–12358
- Meng G, Surana NK, St Geme III JW, Waksman G (2006) Structure of the outer membrane translocator domain of the *Haemophilus influenzae* Hia trimeric autotransporter. *EMBO J* **25**: 2297–2304
- Merritt EA, Bacon DJ (1997) Raster3D: photorealistic molecular graphics. *Meth Enzymol* **277**: 505–524
- Mock M, Pugsley AP (1982) The BtuB group col plasmids and homology between the colicins they encode. *J Bacteriol* **150**: 1069–1076
- Mueller P, Rudin DO, Tien HT, Wescott WC (1962) Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. *Nature* **194**: 979
- Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallograph B* **53**: 240–255
- Neupert W, Herrmann JM (2007) Translocation of proteins into mitochondria. *Annu Rev Biochem* **76**: 723–749
- Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**: 593–656
- Nikaido H, Vaara M (1985) Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**: 1–32
- Ohno-Iwashita Y, Imahori K (1980) Assignment of the functional loci in colicin E2 and E3 molecules by the characterization of their proteolytic fragments. *Biochemistry* **19**: 652–659
- Oomen CJ, van Ulsen P, van Gelder P, Feijen M, Tommassen J, Gros P (2004) Structure of the translocator domain of a bacterial autotransporter. *EMBO J* **23**: 1257–1266
- Osborne AR, Rapoport TA, van den Berg B (2005) Protein translocation by the Sec61/SecY channel. *Annu Rev Cell Dev Biol* **21**: 529–550
- Otwinski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Meth Enzymol* **276**: 307–326
- Pfanner N, Wiedemann N, Meisinger C, Lithgow T (2004) Assembling the mitochondrial outer membrane. *Nat Struct Mol Biol* **11**: 1044–1048
- Phale PS, Philippsen A, Widmer C, Phale VP, Rosenbusch JP, Schirmer T (2001) Role of charged residues at the OmpF porin channel constriction probed by mutagenesis and simulation. *Biochemistry* **40**: 6319–6325
- Roux B, Allen T, Berneche S, Im W (2004) Theoretical and computational models of biological ion channels. *Q Rev Biophys* **37**: 15–103
- Sauri A, McCormick PJ, Johnson AE, Mingarro I (2007) Sec61alpha and TRAM are sequentially adjacent to a nascent viral membrane protein during its ER integration. *J Mol Biol* **366**: 366–374
- Schindler H, Rosenbusch JP (1981) Matrix protein in planar membranes: clusters of channels in a native environment and their functional reassembly. *Proc Natl Acad Sci USA* **78**: 2302–2306
- Sharma O, Yamashita E, Zhalnina MV, Zakharov SD, Datsenko KA, Wanner BL, Cramer WA (2007) Structure of the complex of the colicin E2 R-domain and its BtuB receptor. The outer membrane colicin translocon. *J Biol Chem* **282**: 23163–23170
- Shi Z, Chak KF, Yuan HS (2005) Identification of an essential cleavage site in ColE7 required for import and killing of cells. *J Biol Chem* **280**: 24663–24668
- Smart OS, Goodfellow JM, Wallace BA (1993) The pore dimensions of gramicidin A. *Biophys J* **65**: 2455–2460
- Soelaiman S, Jakes K, Wu N, Li C, Shoham M (2001) Crystal structure of colicin E3: implications for cell entry and ribosome inactivation. *Mol Cell* **8**: 1053–1062
- Taylor R, Burgner JW, Clifton J, Cramer WA (1998) Purification and characterization of monomeric *Escherichia coli* vitamin B12 receptor with high affinity for colicin E3. *J Biol Chem* **273**: 31113–31118
- Tieleman DP, Berendsen HJ (1998) A molecular dynamics study of the pores formed by *Escherichia coli* OmpF porin in a fully hydrated palmitoylphosphatidylcholine bilayer. *Biophys J* **74**: 2786–2801
- Van den Berg B, Clemons Jr WM, Collinson I, Modis Y, Hartmann E, Harrison SC, Rapoport TA (2004) X-ray structure of a protein-conducting channel. *Nature* **427**: 36–44

- Varma S, Chiu SW, Jakobsson E (2006) The influence of amino acid protonation states on molecular dynamics simulations of the bacterial porin OmpF. *Biophys J* **90**: 112–123
- Varma S, Jakobsson E (2004) Ionization states of residues in OmpF and mutants: effects of dielectric constant and interactions between residues. *Biophys J* **86**: 690–704
- Weiss MS, Schulz GE (1992) Structure of porin refined at 1.8 Å resolution. *J Mol Biol* **227**: 493–509
- Wickner W, Schekman R (2005) Protein translocation across biological membranes. *Science* **310**: 1452–1456
- Zakharov SD, Eroukova VY, Rokitskaya TI, Zhalnina MV, Sharma O, Loll PJ, Zgurskaya HI, Antonenko YN, Cramer WA (2004) Colicin occlusion of OmpF and TolC channels: outer membrane translocons for colicin import. *Biophys J* **87**: 3901–3911
- Zakharov SD, Zhalnina MV, Sharma O, Cramer WA (2006) The colicin E3 outer membrane translocon: immunity protein release allows interaction of the cytotoxic domain with OmpF porin. *Biochemistry* **45**: 10199–10207
- Zhang S, Udho E, Wu Z, Collier RJ, Finkelstein A (2004) Protein translocation through anthrax toxin channels formed in planar lipid bilayers. *Biophys J* **87**: 3842–3849