OmpF enhances the ability of BtuB to protect susceptible *Escherichia coli* cells from colicin E9 cytotoxicity

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Abstract The outer membrane (OM) vitamin B_{12} receptor, BtuB, is the primary receptor for E group colicin adsorption to *Escherichia coli*. Cell death by this family of toxins requires the OM porin OmpF but its role remains elusive. We show that OmpF enhances the ability of purified BtuB to protect bacteria against the endonuclease colicin E9, demonstrating either that the two OM proteins form the functional receptor or that OmpF is recruited for subsequent translocation of the bacteriocin. While stable binary colicin E9–BtuB complexes could be readily shown in vitro, OmpF-containing complexes could not be detected, implying that OmpF association with the BtuB–colicin complex, while necessary, must be weak and/or transient in nature.

Key words: BtuB; OmpF; Porin; Bacteriocin; Colicin translocation; Protein–protein interaction

1. Introduction

In the continual battle for survival in nutrient-limited environments, Gram-negative bacteria such as *Escherichia coli* frequently release potent toxins that can selectively eliminate competing bacterial strains but not producing cells [1,2]. Prominent amongst these toxins are colicins, plasmid-encoded protein antibiotics induced via the SOS response. Colicins have evolved to parasitise various cell surface receptors that are normally involved in the uptake and passage of small nutrient molecules, such as iron complexes [3], vitamin B_{12} [4] and nucleosides [5], across the outer cell membrane. The importance of colicins to bacterial colonisation is evident from the diverse range of microorganisms where similar toxins have been identified, including *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Photorhabdus luminescens*, *Shigella flexneri*, *Yersinia pestis* and *Serratia marcescens*.

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Abbreviations: OM, outer membrane; LIS, lithium diiodosalicylate; OG, *n*-octyl-β-D-glucopyranoside; LPS, lipopolysaccharide; Free colicin E9, colicin from which its immunity protein Im9 has been removed; Colicin E9 T-R, truncated colicin in which the C-terminal endonuclease domain has been deleted

Colicins are classified according to the cell surface receptor to which they bind [6]. The E colicins (colicins E1–E9) and colicin A adsorb to a minor component of the outer membrane (OM) – the 66 kDa BtuB receptor protein – which is an essential element of the high-affinity uptake system for cobalamin (vitamin B_{12}) in *E. coli* [7]. It is of interest to note that while all BtuB receptors in the OM have the ability to transport vitamin B_{12}, only a few appear to facilitate colicin action [8]. Translocation of colicins across the cell envelope to their target is poorly understood, but known to be mediated by a group of membrane and periplasmic proteins – the Tol [9] and Ton [10] systems. Cell death by group A colicins (which include colicins A, N and E1–E9) [11] requires the porin OmpF and the Tol system (composed of TolA, B, Q and R proteins), whereas group B colicins (B, D, Ia, M and V) require the Ton system [12]. Both Ton and Tol proteins are responsive to the proton motive force across the inner membrane which, in the case of the Ton system, is responsible for energy-dependent transport of metabolites across the OM [13].

The lethal action of E colicins on susceptible *E. coli* cells requires three events: receptor binding, membrane translocation, and cytotoxicity (reviewed in [2]). Three functional domains of the E colicin proteins have been implicated in each stage of the killing process [14]. Receptor binding occurs via the central 'R' domain, translocation is mediated via the N-terminal 'T' domain, and cytotoxic activity is centred on the carboxy-terminal 'C' domain [2,15]. The toxic effects of group A colicins can be ascribed to one of three activities: colicins A and E1 form pores in the cytoplasmic membrane resulting in collapse of membrane potential (reviewed in [16]); colicins E2, E7, E8 and E9 each possess DNase activity [2,17]; and colicins E3, E4, E5 and E6 are all RNases [2,18]. Colicin-producing bacteria arm themselves against suicide through the co-synthesis of a small (<10 kDa) immunity protein that binds and neutralises the cytotoxic domain with high affinity [2,19]. In the case of 60 kDa enzymatic colicins, the immunity protein associates with the toxin in the producing organism, the heterodimeric complex is then released into the extracellular medium and the immunity protein jettisoned upon adsorption of the colicin complex to its primary receptor [20].

Previous, sometimes contradictory, work on the susceptibility of bacteria toward E group colicins has suggested that adsorption of the toxin requires the presence of monomeric BtuB and the trimeric, major OM protein OmpF [21,22]. Using the pore-forming colicin A in cell killing assays, Chai et al. [22] implicated OmpF, BtuB and lipopolysaccharide (LPS) as

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the functional colicin A receptor. Subsequent work by Benedetti et al. [21] compared the binding of colicins A, E2 and E3 and suggested that OmpF alone was required for both receptor binding and translocation of colicin A, whereas an intermediate situation existed for the enzymatic colicins E2 and E3 where only BtuB acted as receptor but both BtuB and OmpF (along with the Tol system) were required for translocation [21].

To our knowledge there are currently no in vitro data on the role of OmpF in the uptake of E group colicins. We present the first in vitro analysis using purified proteins, showing that OmpF in concert with BtuB is likely involved in forming the functional OM conduit for E group colicins.

2. Materials and methods

2.1. Plasmids, bacterial strains and growth conditions

Wild-type BtuB was overexpressed in the porin-deficient *E. coli* strain TNE012 (K12 *tsx*$^-$*ompA*$^-$*ompB*$^-$) [23,24] from plasmid pNP278. Polymerase chain reaction mutagenesis was used to introduce an *Nco*I restriction site after the signal sequence of BtuB and an *Xho*I restriction site immediately downstream of the stop codon. The 1.8 kb product, amplified from the template pAG1, was cloned into the *Sma*I restriction site of pUC18 to produce pNP271. Plasmid pNP271 was digested with *Nco*I and *Xho*I, and the BtuB gene minus its signal sequence was cloned into complementary sites of pBAD mycC HisB (Invitrogen) to form the recombinant plasmid, pNP278, that was under the tight control of the arabinose inducible promoter.

BtuB synthesis was induced by addition of 0.15% (w/v) L-(+)-arabinose to growing cultures at 37°C with shaking in Luria–Bertani (LB) broth supplemented with 100 μg ml^{-1} ampicillin at an OD_{600} of 0.4–0.6. After induction, cells were grown to an OD_{600} of ~1.5, then harvested by centrifugation and lyophilised to dryness. Lyophilised cells were stored at −20°C until further use.

E. coli strain B^{E}3000 cells were used as a source of OmpF porin [26]. These cells were grown at 37°C with shaking to an OD_{600} of ~1.3 in LB broth supplemented with 0.25% (w/v) D-glucose, then harvested by centrifugation and stored at −20°C until required.

Overproduction of colicin E9, growth of colicin-sensitive JM83 cells for use in cell protection assays and construction of colicin E9 T-R domain has been described elsewhere [27,28].

2.2. Protein purification

BtuB was purified using a protocol modified from that described by Jalal and van der Helm [29] for purification of the FepA receptor protein. 0.5 g of lyophilised *E. coli* TNE012(pNP278) cells (equivalent to ~1.5 l of cell culture) was resuspended in 70 ml of buffer A (10 mM Tris–HCl, pH 8.0, 0.25% lithium diiodosalicylate (LIS) and protease inhibitor cocktail tablets (Roche Diagnostics)), then sonicated on ice. The sonicated cells were centrifuged at 8000×*g* for 10 min at 4°C to pellet any unbroken cells and cell debris. The supernatant was retained and centrifuged for 1 h at 200k×*g*. To solubilise the inner membrane protein fraction, the resulting pellet was resuspended and homogenised in ~56 ml of buffer B (buffer A and 2% Triton X-100), stirred at room temperature for 30 min and centrifuged again at 200k×*g* for 1 h at 20°C. To ensure that all the inner membrane proteins were solubilised, the extraction with buffer B was repeated. BtuB was solubilised by extracting the pellet three times with buffer C (buffer B and 5 mM EDTA, pH 8.0), then a further two times with buffer D (buffer C and 1% (w/v) *n*-octyl-β-D-glucopyranoside (OG) (Melford Chemicals)). The five BtuB-containing supernatants (from extractions with buffers C and D) were pooled and dialysed overnight at room temperature against 3 l of buffer E (50 mM Tris–HCl, pH 7.5, 0.25% LIS, 2% Triton X-100 and 5 mM EDTA) using 25 kDa MWCO membrane (Spectrum Laboratories).

After dialysis the protein solution was loaded onto a 50 ml DE-52 (Whatman) anion exchange column that had been equilibrated in buffer E. After a 50 ml wash, the protein was eluted with a 0–800 mM NaCl gradient. The collected fractions were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% polyacrylamide) using a Hoefer Mighty Small gel apparatus and the BtuB-containing fractions pooled and dialysed overnight against 3 l of 50 mM Tris, pH 7.5, 5 mM EDTA, 0.05% LIS and 2% Triton X-100 using 25 kDa MWCO dialysis membrane.

Detergent exchange and LIS removal were achieved by loading the dialysed protein solution onto a 10 ml DE-52 anion exchange column equilibrated with 100 ml of buffer F (50 mM Tris, pH 7.5, 5 mM EDTA, 1% (w/v) OG). The protein was washed with 50 ml of buffer F and eluted with a 0–800 mM NaCl gradient. Fractions containing BtuB were pooled, desalted and concentrated to ~2 ml using Centriprep YM-50 centrifugal filtration devices (Millipore). The protein was exchanged into 15 mM NaH_2PO_4, pH 7.5, 1% (w/v) OG by passage down a Sephadex G-15 (Pharmacia) gel filtration column.

OmpF porin was purified from *E. coli* B^{E}3000 cells as reported by Nikaido [26]. Purification of free colicin E9 (colicin with the immunity protein removed) and colicin E9 T-R has been described previously [27,28].

2.3. Protein determinations

BtuB and trimeric OmpF concentrations were determined spectroscopically on a Unicam 8625 UV–vis spectrometer using theoretical extinction coefficients (calculated using the ExPASy programme ProtParam tool) at 280 nm of 136 690 and 145 500 M^{-1} cm^{-1}, respectively. The Bradford colorimetric assay was used to determine colicin E9 and colicin E9 T-R domain concentrations.

2.4. Characterisation of BtuB

The secondary structure content of the purified BtuB receptor was characterised by far UV-CD (circular dichroism) spectroscopy (260–190 nm) on an Applied Photophysics π* spectropolarimeter using a 0.1 cm quartz cuvette and a protein concentration of 0.05 mg ml^{-1} (in 15 mM NaH_2PO_4, pH 7.5, 1% (w/v) OG). The spectrum was measured using 1 nm steps with a 25 μs sampling period and both excitation and emission slits were set to 2.0 nm. All measurements were conducted at 20°C under constant nitrogen flush. The secondary structure content of purified BtuB was calculated using the deconvolution software CDNN version 2.1 (http://bioinformatik.biochemtech.uni-halle.de/cdnn/).

Conformation of the receptor was evaluated qualitatively by differential migration (± heating of samples) on SDS–PAGE using a PhastGel system (Pharmacia) essentially as described by Taylor et al. [24].

The ability of purified BtuB to bind vitamin B_{12} was assayed by applying the purified protein to a 2 ml column of vitamin B_{12} immobilised on 4% beaded agarose (Sigma Chemical). 500 μl of a 1.2 mg ml^{-1} solution of BtuB was applied to the column which had previously been equilibrated with 15 mM NaH_2PO_4, pH 7.5, 1% (w/v) β-OG. The column was then washed extensively with the same buffer/detergent solution to remove any unbound receptor. Bound receptor was eluted with a 4 mg ml^{-1} vitamin B_{12} solution and fractions analysed using a 12% SDS–PAGE gel.

2.5. Cell protection assays

Cell protection assays were performed essentially as described by Wallis et al. [30] but with some modifications. 80 μl of colicin-susceptible *E. coli* JM83 cells in mid-log phase was added to 50 ml of 0.7% (w/v) molten micro-agar (Duchefa) at 42°C and used to overlay a 243 mm×243 mm×18 mm square plate containing LB agar supplemented with 100 μg ml^{-1} ampicillin. After drying, 6 μl spots of 2.5 nM free colicin E9 (2 μl of 7.5 nM colicin E9 and 4 μl 15 mM NaH_2PO_4, pH 7.5, 0.85% (w/v) OG) were applied to one lane of the plate as a control. Subsequently, colicin E9 was incubated for 30 min at room temperature with either bovine serum albumin (BSA), BtuB or OmpF (2 μl of 7.5 nM colicin E9, 2 μl receptor and 2 μl buffer) and then spotted onto the relevant lanes of the assay plate. The final receptor concentrations were varied from 125 nM to 1.25 μM to give a range of receptor:colicin E9 from 50:1 to 500:1. The experiment was repeated using equimolar concentrations of BtuB and OmpF or BtuB and BSA. The plate was then incubated overnight at 37°C.

2.6. Gel filtration and cross-linking experiments

Complex formation between the receptors BtuB and OmpF, and free colicin E9 or colicin E9 T-R domain were assayed using size-exclusion chromatography and chemical cross-linking experiments. Colicin E9 T-R domain was chosen as the ligand for the gel filtration experiments because (i) it has an M_r that is sufficiently different from that of either OmpF or BtuB, thereby increasing the resolution of the

gel filtration assay, and (ii) it has been previously shown to bind to BtuB-expressing cells [28].

A pre-packed Sephacryl S-300 HiPrep 16/60 column (Pharmacia) was connected to a Pharmacia FPLC system and equilibrated with 15 mM NaH_2PO_4, pH 7.5, 150 mM NaCl and 1% (w/v) OG at a flow rate of 0.5 ml min^{-1}. Samples of the proteins to be assayed were prepared for chromatography by dialysing them extensively against the same buffer. For each analytical run the final concentration of each protein component was adjusted to 10 μM in a volume of 100 μl. Solutions containing more than one protein component were pre-incubated at room temperature for 30 min prior to application to the column.

Cross-linking used both lipophilic and aqueous cross-linkers and included ethylene glycobis(succinimidylsuccinate) (Pierce), sulfo-EGS, 1-ethyl-3-[3-(dimethylamino)propyl] (Pierce), glutaraldehyde (Sigma Chemical) and the photoreactive cross-linker Tris(2,2′-bipyrydyl)dichlororuthenium(II)hexahydrate ($Ru(bpy)_3Cl_2$) (Aldrich). Typically, reactions were carried out in a total volume of 20 μl consisting of 10 μM protein (in 15 mM NaH_2PO_4, pH 7.5, 150 mM NaCl, 1% (w/v) OG) and cross-linker at a final concentration of 3 mM. The reaction was allowed to proceed for 30 min at 25°C prior to quenching by addition of Tris–HCl, pH 7.5, to a final concentration of 50 mM. After quenching, the reaction mixtures were analysed by SDS–PAGE using pre-cast 4–20% acrylamide gradient gels (Gradipore) and protein bands were visualised by staining with Coomassie blue. In the case of $Ru(bpy)_3Cl_2$ and glutaraldehyde, cross-linking experiments were carried out according to the methods of Fancy and Kodadek [31] and Margosiak et al. [32], respectively.

3. Results and discussion

3.1. Purification and characterisation of BtuB

The purification of BtuB has been described previously [23–25]. After several unsuccessful attempts at the first reported purification of recombinant BtuB [24], which in our hands gave very low yields of protein, we developed a purification scheme based on that used for the iron siderophore porin FepA [29] (see Section 2). Our purification scheme also utilised a novel overexpression construct in which the *btub* gene was placed under the control of an arabinose-inducible promoter and shown previously to produce functional BtuB on *E. coli* cell surfaces [28]. The far UV-CD spectrum of OG-solubilised BtuB receptor purified by this protocol is shown in Fig. 1a. BtuB contains 22 β-strands based on its primary sequence similarity with other large OM porins such as FepA, FecA and Cir, and confirmed by the recently published structure for BtuB [33]. Analysis of the CD of spectrum of purified BtuB (see Section 2) indicated the presence of ~51% β-sheet, in good agreement with the crystallographically determined β-structure content of FhuA [34] and FepA [35] receptors but different from that reported by the earlier study of Taylor et al. for BtuB [24]. Comparison with the amount of β-sheet determined from the structure of BtuB itself awaits release of the pdb coordinates. We assessed the functional status of our purified BtuB preparations using anomalous migration in SDS gels (native BtuB is resistant to SDS-induced denaturation [24]) and its ability to bind to a vitamin B_{12} column (Fig. 1b,c). These indicated that our purified BtuB was natively folded and functionally active.

3.2. OmpF enhances the ability of BtuB to protect cells against colicin E9

Direct involvement of OmpF in E colicin uptake has been inferred but not shown directly. Therefore, to investigate any interactions between the receptors and/or E colicins, cell protection assays were performed using the DNase colicin E9 pre-incubated with BtuB, OmpF, and BtuB in the presence of

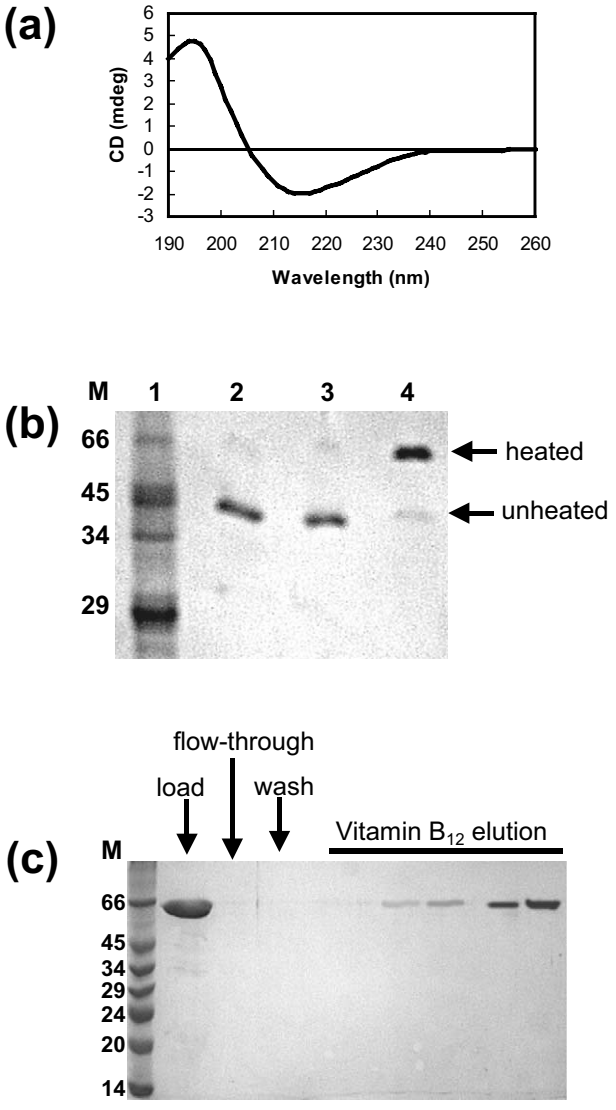

Fig. 1. a: Far UV-CD spectrum of purified BtuB in 15 mM NaH_2PO_4, pH 7.5, 1% (w/v) OG. b: Homogeneous 12.5% Phastgel SDS–PAGE showing anomalous migration of native BtuB. Lane 1, molecular weight markers; lanes 2 and 3, unheated BtuB (apparent M_r ~45 kDa); lane 4, heat-treated BtuB (which migrates at its true M_r of ~66 kDa). c: 12% SDS–PAGE gel showing fractions eluted from a vitamin B_{12} agarose column. The first lane contains molecular weight markers. The second lane shows the BtuB load. The following two lanes represent the flowthrough from the column and the fractions from the wash step, respectively. BtuB receptor eluted when the column was washed with an excess of vitamin B_{12} solution.

OmpF. As can be seen in Fig. 2, colicin E9 pre-incubated with BtuB resulted in complete cell protection being conferred at a BtuB:colicin E9 ratio of ~400:1. This is in contrast with the results obtained by Taylor et al. [24], who reported protection of susceptible cells from the effects of the RNase colicin E3 when the purified receptor was present in only modest stoichiometric excess. The reasons for this disparity may lie in the different ways the cell protection assays were conducted, differing strain sensitivities and/or the different cytotoxic activities of the colicins used.

In the presence of equimolar quantities of OmpF with respect to BtuB, the protection afforded by BtuB is clearly en-

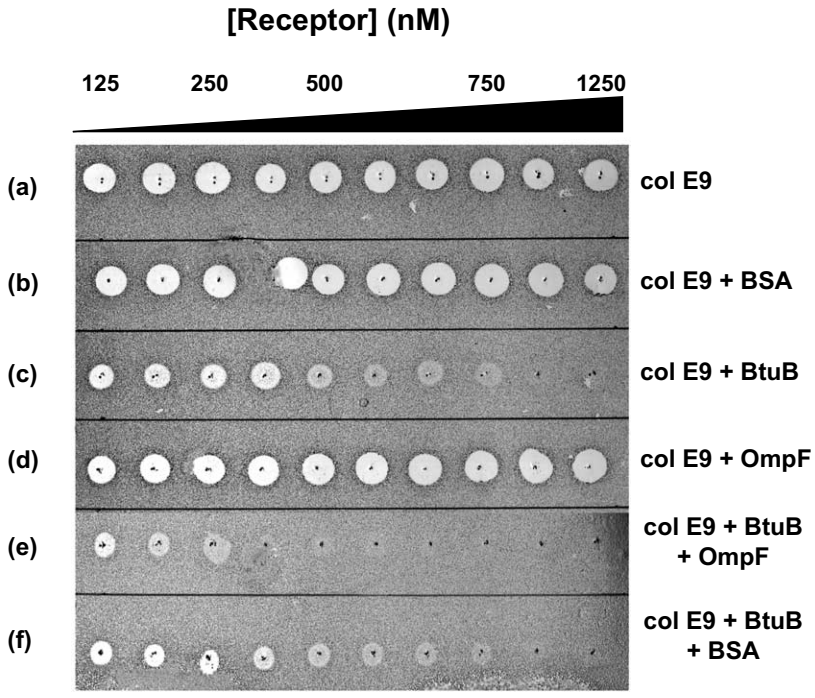

Fig. 2. Plate assay showing the enhancement of protection of susceptible *E. coli* cells provided by BtuB in the presence of OmpF. In all the lanes, colicin E9 was present at a final concentration of 2.5 nM. At this concentration large clear zones, indicating cell death, are visible (lane a). The presence of BSA or OmpF with colicin E9 did not offer any cell protection, indicating that OmpF alone did not bind the colicin (lanes b and d, respectively). Lane c shows the protective effects of BtuB. The same pattern of protection was observed in lane f, in which BSA was present along with BtuB and colicin E9. However, when BtuB was added to colicin E9 in the presence of OmpF cell protection was greatly enhanced (lane e). All protein solutions were prepared in 15 mM NaH_2PO_4, pH 7.5, 0.85% (w/v) OG.

hanced (Fig. 2, lane e). In this case complete protection was conferred at a BtuB/OmpF:colicin E9 ratio of ~200:1. However, as was the case with BtuB incubated with colicin E9, there is a degree of protection conferred at a BtuB/OmpF:colicin E9 ratio of 50:1. This is the first direct evidence that OmpF acts synergistically with BtuB in binding of an enzymatic colicin. To determine if this was specific to OmpF, BtuB was pre-incubated with BSA as a negative control (Fig. 2, lane f). In this case no enhancement of cell protection was observed. This indicates that protein concentration alone was not responsible for the observed enhancement of protection when OmpF was present with BtuB. Hence it seems that the protection afforded by OmpF is due to a specific interaction(s) between OmpF and BtuB or an interaction that is mediated by the colicin.

Previous studies that have suggested OmpF is an important component of the OM receptor for colicin A also suggested that LPS is involved [22]. In our studies, addition of exogenous LPS from *E. coli* K-235 to the receptors produced no change in cell protection (data not shown). However, these and the earlier experiments do not preclude any role for LPS tightly bound to either BtuB (which is known to co-purify with LPS [24,25]) or OmpF. Therefore, the issue of LPS involvement will require closer scrutiny.

3.3. *OmpF associates only weakly or transiently with a BtuB–colicin E9 complex*

Since the cell protection assays had shown OmpF enhanced the protection afforded by BtuB against colicin E9 toxicity we sought more direct methods of establishing the presence of higher-order complexes, focusing on gel-filtration chromatography and chemical cross-linking. For these experiments we used both full-length colicin E9 (from which its immunity protein had been removed) and a truncated version called colicin E9 T-R in which the C-terminal, 15 kDa DNase domain had been deleted. Colicin E9 T-R has previously been shown to bind as effectively to BtuB-expressing *E. coli* cells as intact colicin E9 [28], and was used here because its lower molecular weight (47 kDa) allowed for ready assignment of proteins on accompanying gels and column profiles.

Gel filtration chromatography was performed using 10 μM protein solutions in 15 mM NaH_2PO_4, pH 7.5, 150 mM NaCl and 1% (w/v) OG. Individual proteins eluted as single species, their elution volumes corresponding to their relative differences in molecular mass. Fig. 3 shows that OmpF trimer (M_r = 111 000 Da) eluted at a volume of 59 ml (Fig. 3d), the 66 325 Da BtuB receptor eluted at 65.3 ml (Fig. 3a), and colicin E9 T-R eluted at 70.6 ml (Fig. 3b).

Pre-incubation of stoichiometric amounts of BtuB with colicin E9 T-R prior to loading onto the column resulted in a major peak that eluted at 58 ml (Fig. 3c). SDS–PAGE revealed that this peak was a BtuB–colicin E9 T-R complex, with essentially identical results obtained for full-length colicin E9 (data not shown). When BtuB was pre-incubated with OmpF and the sample ran on the column no stable complex was formed, confirmed by SDS–PAGE analysis of the collected peak fractions (data not shown). Pre-incubation of colicin E9 T-R with a solution of BtuB and OmpF (to give a 1:1:1 stoichiometry) did not result in a supershift of the BtuB–colicin E9 T-R complex (expected M_r of ~248 000 Da) but merely resulted in a peak eluting at 59.5 ml, close to that for the BtuB–colicin E9 T-R complex (Fig. 3e).

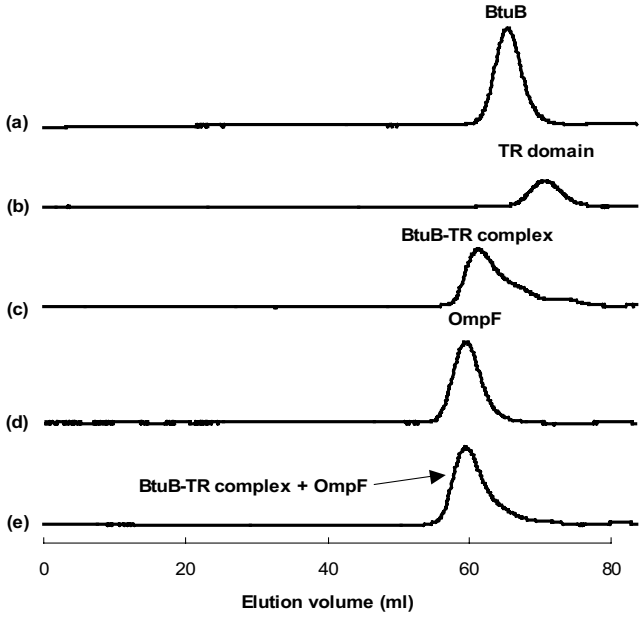

Fig. 3. Gel filtration chromatography was used to monitor complex formation between the BtuB and OmpF receptors, and colicin E9 T-R domain. All protein solutions were prepared in 15 mM NaH_2PO_4, pH 7.5, 150 mM NaCl, 1% (w/v) OG. BtuB eluted at 65.3 ml (panel a), and colicin E9 T-R construct (panel b) and OmpF alone (panel d) eluted at 70.6 ml and 59.2 ml, respectively. Complex formation between BtuB and colicin E9 T-R (10 μM of each protein) could be observed by the presence of a 58.3 ml elution peak (panel c). Under the conditions employed in this assay, no complex formation between BtuB and OmpF, OmpF and colicin E9 T-R domain (data not shown), or BtuB–colicin E9 T-R complex and OmpF (panel e), could be observed.

Chemical cross-linking, using a range of lipophilic and aqueous cross-linking agents (see Section 2), was also used to analyse higher-order complexes. Complexes of colicin E9 and its immunity protein Im9, and BtuB–colicin E9, as well as dimers and trimers of OmpF could be detected in control experiments (data not shown). However, no cross-linked adducts containing both OmpF and BtuB (or BtuB–colicin E9) could be found, which along with the absence of stable complexes on gel-filtration suggests that any interaction between OmpF and the BtuB–colicin E9 complex must be weak or transient in nature or perhaps requires stabilisation by another component of the pathway.

3.4. Conclusions

Very little has previously been published on the role of OmpF in E group colicin adsorption although studies have suggested a role for this receptor in cell killing [21,22]. Through cell protection assays we demonstrate for the first time that OmpF acts synergistically with BtuB to protect bacteria against the action of colicin E9. This interaction could indicate that OmpF is a component of the receptor apparatus for the colicin. Alternatively, OmpF could be recruited by the colicin following its initial docking to BtuB, in which case its role may have more to do with translocation than receptor recognition, as has been suggested by Benedetti et al. [21]. Whichever interpretation is correct, it is clear that the association of OmpF with the BtuB-colicin binary complex is likely to be weak and transient, as witnessed by our failure to detect tertiary complexes either by gel filtration or chemical cross-linking. Having now established an in vitro system incorporating BtuB and OmpF, future work will focus on dissecting the roles of LPS and Tol proteins in colicin uptake that may eventually lead to reconstitution of an intact colicin 'translocon'.

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