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Immunity Protein Protects Colicin E2 from OmpT Protease

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The endonuclease colicin E2 (ColE2), a bacteriocidal protein, and the associated cognate immunity protein (Im2) are released from producing *Escherichia coli* cells. ColE2 interaction with the target cell outer membrane BtuB protein and Tol import machinery allows the dissociation of Im2 from its colicin at the outer membrane surface. Here, we use *in vivo* approaches to show that a small amount of ColE2–Im2 protein complex bound to sensitive cells is susceptible to proteolytic cleavage by the outer membrane protease, OmpT. The presence of BtuB is required for ColE–Im2 cleavage by OmpT. The amount of colicin cleaved by OmpT is greatly enhanced when ColE2 is dissociated from Im2. We further demonstrate that OmpT cleaves the C-terminal DNase domain of the toxin. As expected, strains that over-produce OmpT are less susceptible to infection by ColE2 than by ColE2–Im2. Our findings reveal an additional function for the immunity protein beside protection of producing cells against their own colicin in the cytoplasm. Im2 protects ColE2 against OmpT-mediated proteolytic attack.

Key words: BtuB protein, colicin, immunity protein, OmpT protease, translocation.

Abbreviations: Col, Colicin; Im, Immunity protein; N-domain, N-terminal domain; R-domain, Central domain; C-domain, C-terminal domain; OM, Outer membrane; IM, Inner membrane.

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Endonucleasic colicins are plasmid-encoded antibacterial proteins released by *Escherichia coli* to kill other closely related bacteria (*1*). They display a common organization of three functional domains, each involved in one of the three stages of cell killing: an N-terminal domain (N-domain) required for translocation across the outer membrane (OM) of the target cell; a central domain (R-domain) necessary for binding to cell surface receptors; and a C-terminal domain (C-domain) responsible for the lethal function (*2*).

Colicins enter the cell by interacting with various proteins in the *E. coli* outer envelope, including an OM receptor, and one or more periplasmic proteins. The E colicins (colicins E1 to E9) bind a minor component of the OM, the BtuB receptor protein, which is an essential element of the vitamin B12 high-affinity uptake system in *E. coli* (*3*). This protein complex then interacts with porins, initiating its transport across the OM (*4*, *5*). Colicin translocation across the OM is mediated by a group of membrane and periplasmic proteins of the Tol or the Ton system (*6*, *7*). Once translocated across the *E. coli* envelope, nuclease colicins are supposed to be processed exposing their nuclease domains in the cytoplasm (*8*, *9*), whereas their first two domains remain bound to receptor and import machinery (*10*).

Colicin-producing cells are protected from colicin's lethal action by simultaneous synthesis of an immunity protein that acts specifically against that colicin. Immunity proteins bind to the nuclease colicin C-terminal domain in the cytoplasm with very high affinity and the resulting protein complex is then released into the extracellular medium (*11*). The immunity protein dissociates from the colicin molecule during the uptake process (*12*).

The major role of immunity proteins specific for nuclease colicins is to protect cells against the colicin produced; however, alternative roles have been postulated. It is thought that the ColD immunity protein prevents proteolytic processing of ColD during the secretion process (*8*) and that the ColE3 immunity protein protects the C-terminal domain of the native ColE3 protein from proteolytic degradation before or during translocation across the cell envelope (*13*). A study in 1990 showed that the OM protease OmpT cleaves ColA and E1 (two pore-forming colicins), and ColE2 and E3 (*14*). Recently, several studies have suggested that DNase colicin immunity proteins remain associated with their cognate colicin bound to its translocon (composed of BtuB and OmpF), suggesting that the release of the immunity protein is not the initial step in the nuclease colicin mode of action, and may be one of the last (*4*, *12*). These findings suggest that colicin immunity proteins protect their cognate colicins from OmpT-mediated proteolytic degradation.

In this article, we test this hypothesis by studying the ColE2–Im2 complex. Consistent with previous findings for ColE1 (*15*), we show that OmpT-mediated cleavage of ColE2 is dependent on ColE2 binding BtuB. We also demonstrate that OmpT cleaves and inactivates the C-terminal domain of ColE2, and as expected, Im2 efficiently protects ColE2 from OmpT-mediated degradation. Finally, strains over-producing OmpT are killed more efficiently when ColE2 is in complex with Im2. We conclude

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that DNase colicin immunity proteins are released at a late stage of colicin action, protecting the DNase domain from proteolytic degradation by OmpT.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Wild-type *E. coli* MC4100 and its *btuB* (*btuB-lacZ*) derivative (*3*), or strains UT5600 (*ompT*) (*16*) were used for ColE2 cleavage assays. The plasmid pBRE2 encodes the wild-type colicin E2 operon (ColE2), pAG1 encodes the gene *btuB* (*17*) and pML19 encodes the gene *ompT* (*18*).

Protein Purification—ColE2–Im2 complex was purified as previously described for ColA (*19*). Free ColE2 was isolated by denaturation of the ColE2–Im2 complex in 100 mM sodium phosphate (pH 6.8), containing 6 M guanidine hydrochloride and separation on a Superose 12 column (Pharmacia) equilibrated in the same buffer. Free ColE2 was refolded by dialysis against 100 mM sodium phosphate, pH 6.8, and its nuclease activity was tested as previously described (*12*).

Protease Assay—Cells were grown in LB to the late log phase ($A_{600} = 1$). One-millilitre samples were centrifuged, and the pellets were washed twice with 1 ml of phosphate buffer (100 mM, pH 6.8) and resuspended in 1 ml of the same buffer. Colicins were incubated with the cell suspension in 100 mM phosphate buffer at 37°C, for the times and at the multiplicity (number of colicin molecules per cell) indicated. Samples were then centrifuged.

Far Western Blot—Cell extracts were transferred to nitrocellulose membranes and the membranes were incubated for 1 h in PBS containing 5% skimmed milk and then overnight at 4°C in the presence of 100 μM ColA–Im2 (*20*). ColA–Im2 has been previously constructed to study ColA pore-formation in planar lipid bilayers. However, the Im2 part of this hybrid protein has been described to recognize and to bind to the ColE2 C-terminal domain transferred in nitrocellulose membranes (*12*). Thus, ColA-Im2 can be used to visualize the ColE2 C-terminal domain in far western blot. Nitrocellulose membranes were washed three times in PBS milk, incubated with anti-ColA antibodies (mAb1C11) for 1 h at room temperature, and then with anti-mouse secondary antibodies labelled with Alexa Fluor 488 dye (Molecular Probes) for 1 h at room temperature.

Colicin Assays—*E. coli* MC4100, UT5600 and UT5600 (pML19) cells were grown in LB medium at 37°C to $A = 0.8$. Cells were washed with 100 mM sodium phosphate buffer (100 mM NaH_2PO_4/Na_2HPO_4, pH 6.8) and suspended in this buffer to an OD_{600} of 1. Colicins (50 μl) diluted in sodium phosphate buffer were added to 50 μl of culture and incubated for 20 min at 37°C with shaking. Sodium phosphate buffer with no colicin protein was used as a control. The concentration of undiluted colicins was estimated to be 0.3 mg/ml. Subsequently, 1.25 ml of LB medium was added and cells were incubated for 2 h at 37°C with shaking. The percentage of surviving cells was estimated from ratio between the OD_{600} for the colicin-treated and control cultures.

Outer and Inner Membranes Preparation—Crude inner membrane (IM) and OM fractions were isolated from 1 l cell cultures with OD_{600} of 1. Cells were lysed with a French pressure cell as previously described (*21*), and intact cells were removed by centrifugation for 15 min at 1930*g* in a SS34 rotor. OM fractions were isolated by centrifugation of the supernatant for 5 min (including the time required for starting the centrifuge) at 40,000 r.p.m. ($g_{av} = 105,900g$) in a 50 Ti rotor (Beckman, USA) at 4°C. IM fractions were obtained by centrifugation of the corresponding supernatant for 90 min at 40,000 r.p.m. in a 50 Ti rotor at 4°C. All membrane fractions were suspended in buffer L (50 mM triethanolamine acetate, pH 7.5; 250 mM sucrose; 1 mM dithiothreitol).

RESULTS

BtuB Is Necessary for ColE2 Degradation by an E. coli Envelope Protease—ColE2–Im2 added to sensitive *E. coli* cells is partially cleaved by the OM protease OmpT (*14*). However, this preliminary observation has not been investigated further. In particular, it is not clear whether ColE2 immunity protein partially protects ColE2 from protease degradation. To address this issue, we added various concentrations of free ColE2 to MC4100 cells and derivative cell lines. Cell extracts and supernatants were then loaded onto SDS–PAGE gels, and colicin protein was detected by immunoblotting with anti-ColE2 antibodies. At a multiplicity (number of colicin molecules per cell) of 1,000 most of the ColE2 protein (62 kDa full-length) recovered from the MC4100 cell pellet had been degraded and converted into a 50 kDa fragment (Fig. 1; upper panel, lane 2). At a multiplicity of 40,000 at least 50% of ColE2 associated with MC4100 cells had been degraded (Fig. 1; upper panel, lane 4); whereas ColE2 recovered from the supernatant of this sample remained intact (Fig. 1; upper panel, lane 4a). We suspected that ColE2 binding to BtuB is necessary for its cleavage by *E. coli* envelope proteases, as has been previously described for the pore-forming

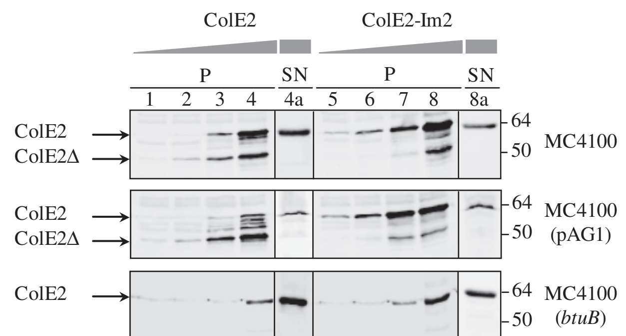

Fig. 1. **Protease susceptibility of ColE2 and the ColE2–Im2 to proteolytic cleavage.** Different multiplicities (*M* = number of colicin molecules per cell) of ColE2 (lane 1, *M* = 200; lane 2, *M* = 1,000; lane 3, *M* = 10,000; lane 4 *M* = 40,000) and ColE2–Im2 (lanes 5–8) were incubated 10 min at 37°C with MC4100 (upper panel), MC4100 (pAG1) (middle panel), and MC4100 (*btuB*) (lower panel) as described in MATERIALS AND METHODS section. pAG1 encodes the BtuB receptor. Cells were harvested by centrifugation and pellets (1 A_{600} units of cells) were loaded onto SDS–PAGE gels (lanes 1–8). The supernatants of samples 4 and 8 (0.05 A_{600} units of the orignal cells suspension) were also loaded onto SDS–PAGE gels (lanes 4a and 8a). Full-length ColE2 (62 kDa) and the large processed fragment ColEΔ (50 kDa) were detected by immunoblotting with anti-ColE2 antibodies.

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ColE1 (*15*). To confirm this, ColE2 was added to MC4100 cells carrying pAG1, which overproduce the BtuB receptor (*17*), or to the MC4100 *btuB* strain which did not produced the BtuB receptor. Most ColE2 added at the multiplicity of 40,000 to MC4100 cells carrying pAG1 was both associated with the cells and degraded (Fig. 1; middle panel, lane 4), whereas ColE2 added to the MC4100 *btuB* strain was mostly recovered in the supernatant in its full-length form (Fig. 1, lower panel, lane 4a). Moreover, the small amount of ColE2 recovered from the MC4100 *btuB* cell pellet was not degraded (Fig. 1; lower panel, lane 4). Thus, the presence of BtuB is required for ColE2 degradation by an *E. coli* envelope protease. Previous results indicated that OmpT, an OM protease, cleaved colicins (*14*). For this reason, we tested the proteolytic activity of OmpT on ColE2 *in vivo*. As expected, cleavage of ColE2 was abolished in the *ompT* mutant but was restored in cells carrying a plasmid-borne copy of *ompT* (Fig. 2).

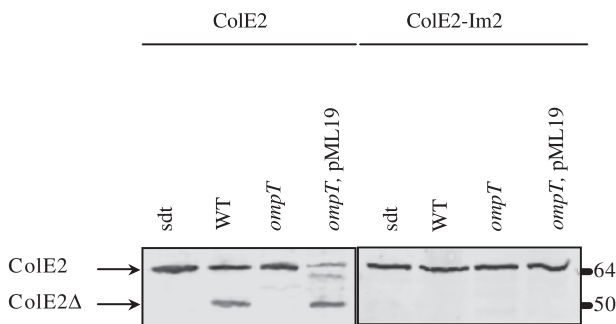

Fig. 2. **OmpT cleaves ColE2.** ColE2 and ColE2-Im2 were incubated 10 min at 37°C with MC4100, UT5600 (*ompT*) and UT5600 (*ompT*, pML19) at a multiplicity of 10,000 as described in MATERIALS AND METHODS section. pML19 encodes the OmpT protease. Full-length ColE2 (62 kDa) and the large processed fragment ColEΔ (50 kDa) were detected by immunoblotting with anti-ColE2 antibodies. 'Sdt' indicates a standard amount of ColE2 and ColE2-Im2 (M = 10,000) loaded alone in the SDS–PAGE and detected by immunoblotting with anti-ColE2 antibodies.

The pattern of proteolytic digestion was not identical to that described by Cavard and Lazdunski in 1990, the main reason being that we used less ColE2 protein in our studies. Even at the multiplicity of 40,000 we added 10-fold less colicin protein per cell than Cavard and Lazdunski in 1990. We observed another cleavage site when more colicin protein was added to the cells, and recovered cleaved colicins from the supernatant (data not shown). However, in both studies, the main product of ColE2 digestion by OmpT was a 50 kDa fragment.

Im2 Protects ColE2 Against Protease Degradation—The heterodimeric complex ColE2–Im2 released into the surrounding medium from ColE2-producing bacteria interacts with the target bacteria. Im2 remains associated with ColE2 during the uptake process until the ColE2 N-terminal domain has been fully translocated (*12*); thus, Im2 could protect the ColE2 C-terminal domain against proteolytic degradation by OmpT. To test this, we added ColE2-Im2 to MC4100 cells as described above. ColE–Im2 added at a multiplicity of 1,000 was resistant to protease degradation, and only a small amount of the complex was degraded when added at a multiplicity of 40,000 (Fig. 1; upper panel, lanes 6–8a). Protein degradation was dependent on BtuB binding, as observed for free ColE2 (Fig. 1; middle and lower panels). Comparison of these findings with those obtained using free ColE2 suggests that Im2 protects ColE2 from OmpT degradation. We studied whether Im2 is able to protect ColE2 over a sustained period of time. To do this, we isolated cell extracts at various times after addition of ColE2 or ColE2–Im2 at a multiplicity of 10,000. ColE2 was degraded as soon as it was added to MC4100; in contrast, ColE2-Im2 was still intact even 60 min after its addition to the cells (Fig. 3A). As expected, ColE2 and ColE2–Im2 were not cleaved, even 60 min after their addition to the *ompT* strain (Fig. 3B). To determine whether Im2 added externally protected ColE2 bound to cells, Im2 was added at various times after ColE2 addition to cells. We observed a time-dependent protection of ColE2 by added Im2 (Fig. 3C).

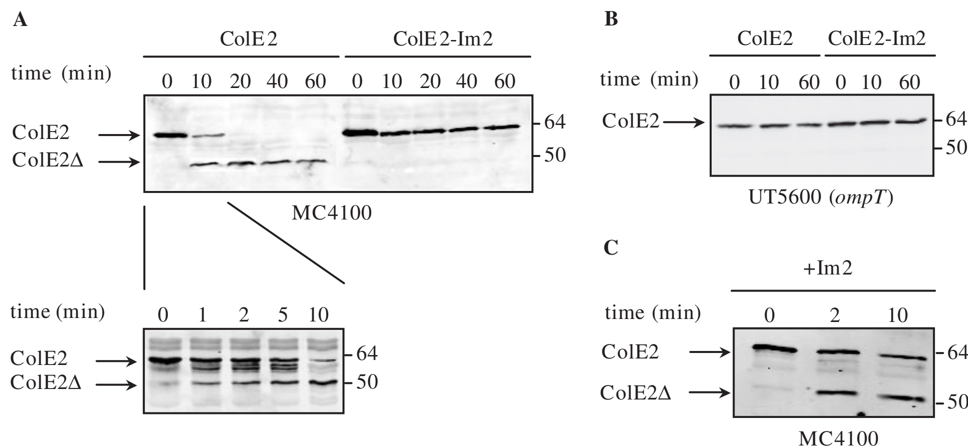

Fig. 3. **Im2 protects ColE2 from OmpT attack.** ColE2 and ColE2-Im2 were incubated at a multiplicity of 10,000, at the indicated times and at 37°C with MC4100 (A) or UT5600 (*ompT*) (B) as described in MATERIALS AND METHODS section. In (C), ColE2 was incubated at a multiplicity of 10,000 with MC4100; Im2 (10-fold excess) was added to the cells at the indicated times. Cells were further incubated 10 min and recovered by centrifugation. Full-length ColE2 (62 kDa) and the large processed fragment ColEΔ (50 kDa) were detected by immunoblotting with anti-ColE2 antibodies.

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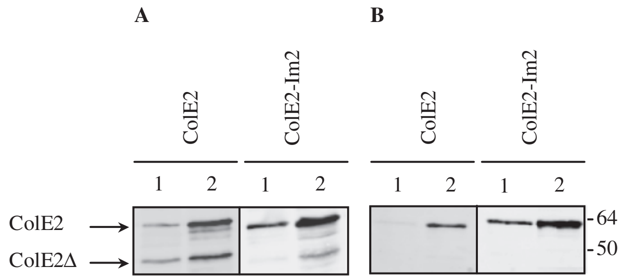

Fig. 4. **OmpT cleaves the DNase domain of ColE2.** ColE2 and ColE2-Im2 were incubated with MC4100 as described above. Cells were harvested by centrifugation and pellets were loaded onto SDS–PAGE. Full-length ColE2 (62 kDa) and the large processed fragment ColEΔ (50 kDa) were detected by immunoblotting with anti-ColE2 antibodies (A) or by overlay method with purified ColA-Im2 (B). The Im2 part of the ColA–Im2 hybrid protein has been described to recognize and to bind to the ColE2 C-terminal domain transferred in nitrocellulose membranes (cf. EXPERIMENTAL PROCEDURES; far western blot).

Im2 protected ColE2 from proteolysis when the two proteins were added simultaneously. When Im2 was added 2 or 10 min after ColE2 addition, Im2 did not protect ColE2 from proteolysis.

OmpT Cleaves the C-Terminal Domain of ColE2—At the colicin concentration used in the experiments above, full-length colicin molecules were converted into a single and smaller fragment of ~50 kDa. We suspected that the region of ColE2 cleaved by OmpT was the DNase domain for two reasons. First, this region is rich in basic residues, and OmpT proteolysis has been reported to take place between pairs of basic residues. Second, Im2, which protects ColE2 against OmpT proteolysis, binds strongly to this domain. To investigate this, we added ColE2 or ColE2-Im2 to MC4100 cells at multiplicities of 1,000 and 40,000. Cell extracts were loaded onto SDS-PAGE gels. The content of each fraction was analysed by immunoblotting with anti-ColE2 or anti-ColA antiserum using the far western-blot method with purified ColA-Im2, which specifically recognizes the C-terminal domain of ColE2 (see MATERIALS AND METHODS section). ColA–Im2 did not recognize the 50 kDa fragment (Fig. 4), suggesting that this fragment does not contain the DNase domain of ColE2. We have previously showed that ColA–Im2 binds CTE2 with nearly the same affinity than Im2 to ColE2 (*20*).

ColE2–Im2 Cleavage by Crude E. coli OM Fractions—ColE2 and ColE2–Im2 were incubated with crude outer and IM fractions and colicin degradation by OmpT was studied. Free ColE2 incubated with OM was converted into the 50 kDa fragment; ColE2–Im2, however, was digested to give three proteolytic fragments with apparent molecular weights of 50, 33 and 18 kDa when incubated with OM (Fig. 5A). In contrast to the 33 and 18 kDa fragments, the 50 kDa fragment was not recognized by ColA–Im2, suggesting that this fragment did not contain the DNase domain. It is likely that the 18 kDa fragment was the DNase domain protected from OmpT digestion by Im2. The presence of a 33 kDa fragment suggests that ColE2–Im2 is cleaved in the middle, probably in the R-domain. Thus, we concluded that the DNase domain of free ColE2 is extensively digested by OmpT, whereas in the presence of Im2 a small amount of the DNase domain is dissociated from the other ColE2 domains. ColE2 and ColE2–Im2 controls remained intact when incubated with purified OM devoid of BtuB (Fig. 5B) or OM devoid of OmpT (Fig. 5C). ColE2 and ColE2–Im2 were not cleaved when incubated with crude IM fractions (Fig. 5).

Cells Expressing OmpT Are Less Susceptible to the Toxic Effects of ColE2—The cleavage of colicins A, E1, E2 and E3 by OmpT may be a defense mechanism used by colicin-sensitive bacteria (*14*). A recent study used a double-layer plate test by cross-streaking *E. coli* strains with ColE1 and ColE2 colicinogenic strains to show that OmpT production was advantageous for a small number of bacteria that survive the initial exposure to the toxin (*15*). As previously described by Bénédétti *et al.* (*22*), we show that free ColE2 was about 10-fold less active than ColE2–Im2 on wt bacterial strain (Fig. 6A). To prove that this loss of killing activity was due to the colicin degradation by OmpT, we performed the same colicin-killing assay on bacterial strain over-producing or not producing OmpT. We showed that ColE2–Im2 and ColE2 killing activities were similar for the *ompT* mutant (Fig. 6B). In contrast, for the strain over-expressing OmpT, free ColE2 had an approximately 50 fold lower activity than ColE2–Im2 (Fig. 6C). Interestingly, ColE2–Im2 killing activity was approximately the same on bacterial strain producing or not OmpT, indicating that Im2 efficiently protected ColE2 from OmpT attack. These findings suggest that OmpT targets the C-terminal domain of, and inactivates, free ColE2.

DISCUSSION

OmpT is a member of the omptin family, a class of proteases that lacks the signature sequences of classical protease families (*23*). Omptin family members—which are commonly found in Gram-negative bacteria—are multifunctional and have recently been implicated in the breakdown of antimicrobial peptides (*24*). Following the initial observation that OmpT mediates the cleavage of pore-forming and nuclease colicins (*14*), Masi *et al.* (*15*) showed that OmpT cleaves the N-terminal domain of ColE1 and suggested that OmpT degrades colicin at the cell surface.

Our study confirms these findings and shows that ColE2 is also susceptible to OmpT cleavage. However, OmpT cleaved the C-terminal domain of ColE2 rather than the N-terminal domain as was shown for the pore-forming ColE1. The recent elucidation of the crystal structure of OmpT revealed a 10-stranded anti-parallel β-barrel that protrudes from the lipid bilayer into the extracellular space, with a proteolytic site located at the extracellular end of the vase shaped β-barrel (*25*). The location of this proteolytic site and the fact that BtuB is required suggest that colicin cleavage occurs on the extracellular surface of the OM. Interaction between the ColE2 R-domain and its BtuB receptor may underpin the dependence of OmpT digestion on BtuB (*26*). Indeed, the recently published structure of the complex revealed that the R-domain is bound to BtuB in an oblique orientation. This orientation allows the N-domain

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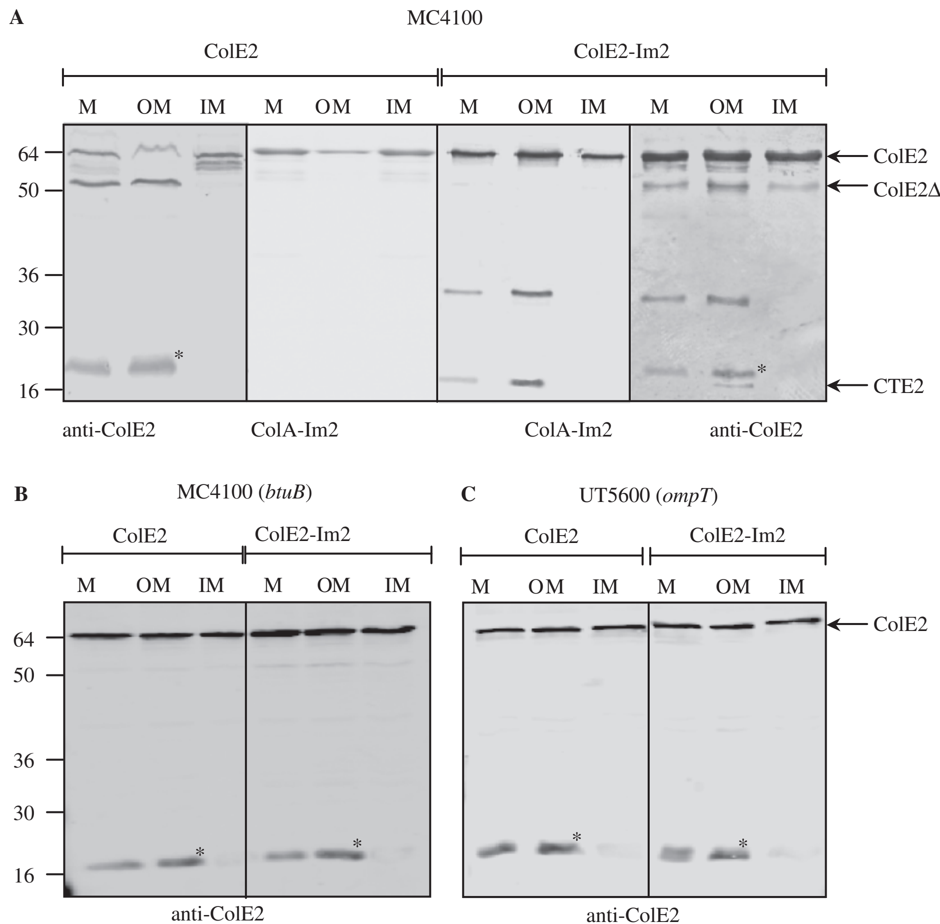

Fig. 5. **ColE2 and ColE2–Im2 incubation with crude OM and IM fractions.** Total membrane (M), outer membrane (OM) and inner membrane (IM) of 5×10^9 cells from MC4100 (A), MC4100 (*btuB*) (B) or UT5600 (*ompT*) (C) were incubated with 3 μg of ColE2 or ColE2–Im2, for 30 min at 37°C. Samples were loaded onto SDS–PAGE gels (18%) and full-length ColE2 and its proteolytic fragments were detected by immunoblotting with anti-ColE2 antibodies or by overlay method with purified ColA–Im2, as indicated in the figure. The peptide indicated by asterisk is an outer membrane contaminant (Roland Lloubès and Denis Duché) recognized by the anti-ColE2 antibodies.

to recruit a porin (OmpF or OmpC) for its translocation through the OM and allows the C-domain to come into contact with OM proteins. Possibly, the C-domain is in close proximity to OmpT during N-domain—OmpF interaction and therefore susceptible to cleavage. OmpT preferentially cleaves protein or peptide substrates between two successive basic amino acid residues or after a basic residue followed by nonpolar amino acid (*24*). The ColE2 C-domain is mainly composed by basic amino acids and possesses a net positive charge of +11 and thus offers a large number of such sites. Consistent with this, we detected multiple proteolytic fragments with sizes between the full-length ColE2 and its major proteolytic fragment of 50 kDa (Fig. 1 middle panel and 3A).

We showed that Im2 prevents the degradation of the E2 DNase domain and considerably inhibits its cleavage by OmpT, presumably by steric hindrance. Indeed, at the initial stage of ColE2 translocation through the *E. coli* envelope, its DNase domain may dynamically interact with at least OmpF, Im2 and OmpT. We hypothesized that the presence of Im2 prevents the interaction between OmpT and E2 DNase domain and therefore the degradation of the E2 DNase domain. A similar observation was made by Zhang *et al*. (*27*), who showed that a modified ColE9 bound to Im9 and incubated with sensitive cells was inaccessible to externally added protease in contrast to ColE9 free of Im9. We previously showed that the dissociation of Im2 from ColE2 occurred at the OM and required full translocation of the ColE2 N-terminal domain, suggesting that release of immunity proteins is not the initial step in the mode of action of nuclease colicin, but one of the last (*12*). We believe that Im2 remains associated to ColE2 DNase domain until it enters the cell protecting ColE2 from OmpT-mediated degradation.

The major proteolytic fragment observed in our experiments had an apparent molecular weight of 50 kDa, and did not include the intact ColE2 DNase domain. However, we showed that the DNase domain is cleaved when ColE2–Im2 is incubated with the OM fraction of sensitive cells. Indeed, several authors have suggested that cleavage of the nuclease domain is an essential step for import and cell killing (*8*, *9*, *26*).

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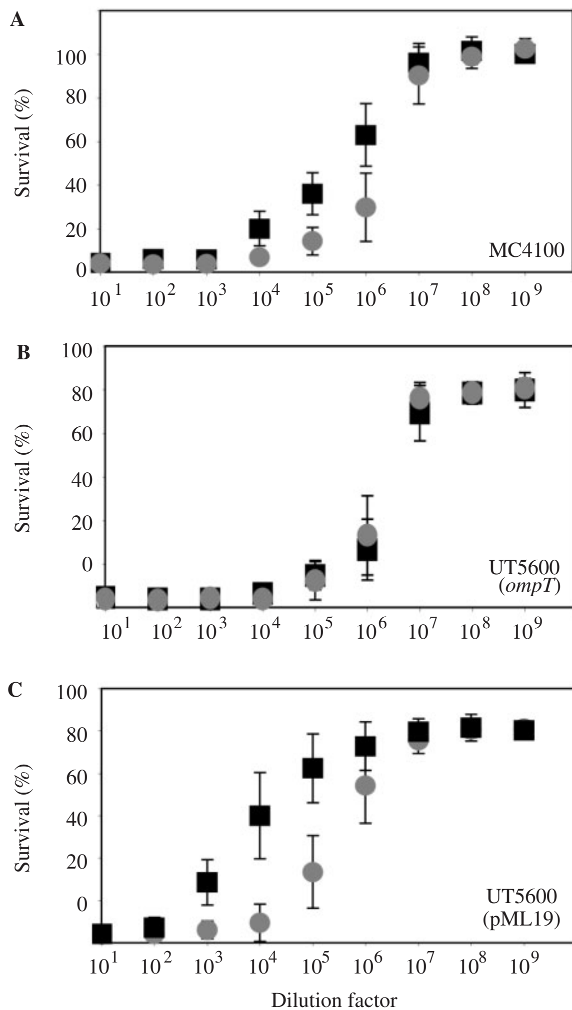

Fig. 6. **Activity assays for ColE2 and ColE-Im2 on cells producing or not producing OmpT.** A volume (50 µl) of ColE2 (filled square) or ColE2-Im2 (filled circle) dilution was added to 50 µl of MC4100 (A), UT5600 (*ompT)* (B) or UT5600 (*ompT,* pML19) (C) as described in MATERIAL AND METHODS and incubated for 20 min at 37°C with shaking. pML19 encodes the OmpT protease. As a control, sodium phosphate buffer was used instead of the colicin dilution. LB medium was then added and the culture was incubated for 2 h at 37°C with shaking. The percentage of viable cells was determined from the OD_{600} ratios of the colicin treated and control cultures. Each assay was done in triplicate.

This proteolytic processing seemed likely to occur in the periplasm or in the OM. However, OmpT is not required for activation of ColE2 because cells without OmpT were efficiently killed by ColE2.

In conclusion, we confirm that OmpT protects cells against colicin attack (*15*). We show that OmpT efficiently cleaves the DNase domain of ColE2 bound to the cells, and demonstrate that Im2 protects the ColE2 DNase domain from OmpT proteolysis. It is clear that the major role of immunity proteins is to protect cells against the colicin produced. Beside this role, it has been hypothesized that immunity protein may prevent colicin processing during the secretion process (*8*). Finally, we propose that immunity proteins protect DNase domains from OmpT-mediated proteolysis until these domains enter the periplasm of the target cell.

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CONFLICT OF INTEREST

None declared.

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