JOURNAL OF BACTERIOLOGY, Dec. 2006, p. 8593–8600 Vol. 188, No. 24
0021-9193/06/$08.00+0 doi:10.1128/JB.00941-06

Release of Immunity Protein Requires Functional Endonuclease Colicin Import Machinery▽

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Received 29 June 2006/Accepted 22 September 2006

Bacteria producing endonuclease colicins are protected against the cytotoxic activity by a small immunity protein that binds with high affinity and specificity to inactivate the endonuclease. This complex is released into the extracellular medium, and the immunity protein is jettisoned upon binding of the complex to susceptible cells. However, it is not known how and at what stage during infection the immunity protein release occurs. Here, we constructed a hybrid immunity protein composed of the enhanced green fluorescent protein (EGFP) fused to the colicin E2 immunity protein (Im2) to enhance its detection. The EGFP-Im2 protein binds the free colicin E2 with a 1:1 stoichiometry and specifically inhibits its DNase activity. The addition of this hybrid complex to susceptible cells reveals that the release of the hybrid immunity protein is a time-dependent process. This process is achieved 20 min after the addition of the complex to the cells. We showed that complex dissociation requires a functional translocon formed by the BtuB protein and one porin (either OmpF or OmpC) and a functional import machinery formed by the Tol proteins. Cell fractionation and protease susceptibility experiments indicate that the immunity protein does not cross the cell envelope during colicin import. These observations suggest that dissociation of the immunity protein occurs at the outer membrane surface and requires full translocation of the colicin E2 N-terminal domain.

Colicins are plasmid-encoded cytotoxins synthesized by *Escherichia coli*, which are secreted into the medium and kill sensitive strains of competing bacteria (4). Four cytotoxic classes of colicin have thus far been identified: the pore-forming colicins such as colicin A (ColA), ColE1, ColN, ColK, ColIa, ColIb, and ColD, which kill cells by causing membrane depolarization (22); RNase colicins, such as ColE3, ColE4, ColE6, and cloacin DF13, which specifically cleave 16S ribosomal (2, 31), or ColD and ColE5, which cleave the anticodon loops of distinct tRNA (27, 34); DNase colicins such as ColE2, ColE7, ColE8, and ColE9, which are nonspecific endonucleases (8, 29, 14); and inhibitors of cell wall synthesis such as colicin M (30). Despite their different lethal activities, colicins display a similar organization of three functional domains, each involved in one of the three stages of cell killing: an N-terminal part required for translocation across the outer membrane (OM), a central domain necessary for binding to a cell surface receptor, and a C-terminal domain encoding the lethal function (1).

Colicins have evolved to parasite various cell surface receptors that are normally involved in the uptake and passage of small nutrients molecules, such as iron complexes, vitamin B_{12}, and nucleosides across the OM (11). For example, the E colicins (colicins E1-E9) and ColA absorb to a minor component of the OM—the BtuB receptor protein—which is an essential element of the high-affinity uptake system for vitamin B_{12} in *E. coli* (28). After binding to specific receptors, colicins are translocated across the OM in a process mediated by a group of membrane and periplasmic proteins, the Tol or the Ton system. Colicins have been classified into two groups (A and B) depending on the translocation system they used to enter the cells. Group A colicins (ColA, E1 to E9) use the Tol system (composed of TolA, -B, -Q, and -R), whereas group B colicins (ColB, -Ia, -Ib, -D, -M, -5, and -10) use the Ton system (TonB, ExbB, and ExbD) (9, 10). Both the Ton and Tol systems are coupled to the proton motive force across the inner membrane, acting as energy transducers for active transport (Ton) or maintenance of OM integrity (Tol) (24). Most colicins also required porins for their transport across the OM. Colicins N and A use OmpF both as receptor and for translocation, whereas colicins E2 to E9 require OmpF, OmpC, or PhoE for translocation.

The enzymatic activity of nuclease colicin needs to be neutralized inside the producing bacterial cell in order to avoid suicide. This is achieved through the action of a small immunity protein (19). Immunity proteins form very tight complexes with nuclease colicins and inhibit their nuclease activity. Im9, for example, interacts with the DNase domain of colicin E9 with an equilibrium dissociation constant of 9.3×10^{-17} M, one the highest affinities known for a protein-protein interaction (38). The heterodimeric complex is then released into the surrounding medium. During the uptake process the cognate immunity protein is dissociated from the colicin molecule, but how and exactly when this occurs is not known (20). Recently, Housden et al. (16) have proposed that immunity protein of ColE9 is not released during association of the bacteriocin complex with its receptor BtuB and OmpF (16).

The major problem in studying the release of the immunity protein is the very small amount of BtuB receptor at the membrane surface (200 to 400 copies per cell [13]). Indeed, at the physiological multiplicity of 400 ColE2-Im2 complexes per cell,

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▽ Published ahead of print on 29 September 2006.

it is difficult to detect the immunity protein by Western blotting because of its low molecular weight and of the lack of high-affinity antibodies. To facilitate detection of the immunity protein, a derivative of the wild-type enhanced green fluorescent protein (EGFP) was fused to its amino terminus. The EGFP derivative has been engineered for enhanced solubility and fluorescence yield compared to the natural protein. We use EGFP-Im2 here in order to gain more insight into the fascinating problem of the colicin-immunity dissociation. We first showed that EGFP-Im2 interacted specifically with unbound ColE2 in a one-to-one molar ratio, resulting in complete inhibition of colicin DNase activity. Added to sensitive cells, the reconstituted ColE2/EGFP-Im2 complex dissociated, and the EGFP-Im2 was released into the culture medium. This prompted us to investigate the involvement of BtuB, OmpF, and the various Tol proteins in the immunity release.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* C600 was used as a recipient strain for all cloning procedures. Strains used for the sensitivity assay were the wild-type MC41000 strain and the *ompF* (*ompF-lacZ*) or *btuB* (*btuB-lacZ*) derivatives (7) or the wild-type C600 strain and the *tolA*, *tolB*, or *tolR* derivatives (6) and *E. coli* SM1005 (*ompC*, *ompF14*) (15). Plasmid pBRE2 encodes the wild-type colicin E2 operon (ColE2), pEGFP encodes EGFP, and pJFIm2 encodes the wild-type Im2. The plasmids pBRE2 and pEGFP were used as a template to amplify the DNA regions encoding Im2 and EGFP, respectively. pBADHisC (Invitrogen) vector was used to fused the DNA region encoding EGFP-Im2 upstream a sequence encoding a His_6 tag.

Constructs. DNA fragments encoding EGFP and Im2 were amplified by PCR (PCRs 1 and 2, respectively) using pEGFP and pBRE2 as a template, respectively. The 3′ primer used for PCR 1 and the 5′ primer used for PCR 2 also encoded for the last codons of EGFP and the first codons of Im2. PCR fragments 1 and 2 were assembled by the overlap method as for the EGFP-Im2 gene. These genes were inserted into the XhoI and HindIII sites of pBADHisC, and the entire construct was sequenced.

Cell fractionation. MC4100 cells containing plasmids encoding EGFP-Im2 were induced with 0.5 mg of arabinose/ml for 30 min at 37°C, or MC4100 cells were grown in LB medium at 37°C to an optical density at 600 nm (OD_{600}) of 0.75 and incubated for 5 or 20 min with ColE2/EGFP-Im2 at a multiplicity of 400. The cells (20 ml) were then pelleted by centrifugation (T) and suspended in spheroplast buffer (0.2 ml, containing 0.2 M Tris-HCl (pH 8.0), sucrose (0.5 M), and EDTA (0.5 mM). A freshly prepared solution (10 μl) of lysozyme (2 mg/ml in H_2O) was added, followed by the addition of spheroplast buffer (0.4 ml) in H_2O (0.4 ml). The cells were gently mixed and then incubated for 20 min at 4°C. Spheroplasts were pelleted by centrifugation at 2,000 × *g* for 10 min. The supernatants (P) corresponded to the periplasm. The pellet was resuspended in spheroplast buffer (1 ml) containing 2 mM phenylmethylsulfonyl fluoride, and the spheroplasts were lysed by three cycles of freezing and thawing. The suspension was centrifuged at 100,000 × *g* for 30 min. The supernatant corresponded to the cytoplasmic fraction (C). The pellet was washed with 1 ml of spheroplast buffer, suspended in 1 ml of spheroplast buffer, and saved as the membrane fraction (M).

Protease accessibility experiments. Cells were grown in LB medium at 37°C to an OD_{600} of 0.75 and incubated for 5 or 20 min with ColE2/EGFP-Im2 at a multiplicity of 400. Trypsin was added to a final concentration of 200 μg/ml for 5 min at 37°C. Then, trypsin inhibitor (150 μg/ml) was added. Cells and supernatant were separated by centrifugation, and the supernatant was concentrated by ultrafiltration. Pellets and supernatant samples were suspended in sodium dodecyl sulfate sample buffer.

Fluorescence microscopy. Cells for fluorescence microscopy were fixed to a poly-lysine film and observed with a 100× oil immersion objective lens. Routinely, fluorescence images were recorded, using an integration time between 40 ms and 8 s as appropriate, and a phase-contrast image of the same field was recorded. The mean pixel intensity was measured as described previously (23).

Overlay method. After Western blotting, the nitrocellulose membranes were incubated for 1 h in phosphate-buffered saline containing 5% skimmed milk and then overnight at 4°C in the presence of 100 μM ColA-Im2 (32). The nitrocellulose membrane were washed three times in phosphate-buffered saline–milk, incubated with antibodies directed against ColA (MAb1C11) for 1 h at room temperature, and then incubated for 1 h at room temperature with anti-mouse second antibodies labeled with Alexa Fluor 488 dye (Molecular Probes).

Assay for nuclease activity in vitro. Protein samples (15 μl) were incubated at 25°C for 30 min in Tris-HCl (50 mM [pH 8.0]) containing NaCl (150 mM) and magnesium sulfate (10 mM). Substrate DNA (5 μl containing 150 ng of linear pUC18) in the same buffer was then added to each reaction mixture and incubated at 37°C for 1 h. The reactions were stopped with loading buffer and electrophoresed in a 1% agarose gel.

Gel filtration experiments. Complex formation between EGFP-Im2 and free colicin E2 was assayed by using size-exclusion chromatography. Samples (40 μM protein solutions in 100-μl aliquots) for gel filtration were applied in 100 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl to a Superdex-200 10/300GL column (M_r fractionation range of 10,000 to 600,000) equilibrated in the same buffer. All proteins were incubated at 25°C for 30 min prior to injection. The flow rate was kept at 0.5 ml/min, and size-exclusion chromatography experiments were performed at room temperature. Elution was monitored by measurement of optical density at 280 nm (OD_{280}). To calibrate the Superdex-200 column, we obtained experimental values for the elution volumes (V_e) of the following standard proteins with known molecular weights: ferritin (440,000 Da; V_e = 9.05 ml), alcohol dehydrogenase (200,000 Da; V_e = 11.17 ml), β-Amylase (150,000; V_e = 11.98 ml), bovine serum albumin (67,000 Da; V_e = 12.80 ml), and ovalbumin (43,000 Da; V_e = 13.47 ml). The volume of eluent at which the individual standard proteins emerged from the column was plotted against the logarithm of their molecular mass and fit by linear regression (correlation coefficient = 0.98).

Protein purifications. ColE2-Im2 complex was purified as described for the purification of the colicin A (12). Free colicin E2 was isolated by denaturation of the colicin E2-Im2 complex in 100 mM sodium phosphate (pH 6.8) containing 6 M guanidine hydrochloride and separated on a Superose-12 column (Pharmacia) equilibrated in the same buffer. Free colicin E2 was refolded by dialysis into 100 mM sodium phosphate (pH 6.8). EGFP-Im2 was expressed from *E. coli* strain MC4100 harboring the plasmid pBAD-EGFP-Im2 and purified by metal affinity chromatography. Two liters of LB broth (100 μg of ampicillin/ml) was inoculated with 40 ml of an overnight culture of *E. coli* MC4100 containing pBAD-EGFP-Im2, followed by incubation at 28°C with shaking. At an absorbance (600 nm) of 0.8, the expression of EGFP-Im2 was induced by the addition of L-arabinose at a final concentration of 0.5 mg/ml for 16 h. The culture was centrifuged for 20 min at 10,000 × *g*. Cells were resuspended in 40 ml of ice-cold 100 mM phosphate buffer containing, NaCl (300 mM) and the protease inhibitor phenylmethylsulfonyl fluoride (1 mM) (buffer A) and broken by using a French press at 900 to 1,000 lb/in^2. After incubation with 0.3 mg each of DNase and RNase (at 37°C for 30 min), the cell debris was removed by centrifugation, and the supernatant was dialyzed against buffer A. Protein was isolated by using TALON metal-affinity resin as described by the supplier (Clontech). Protein concentrations were measured against a series of bovine serum albumin standards by using the Bradford protein assay and are expressed as average values from at least three independent replicates.

RESULTS

Synthesis of EGFP-Im2 hybrid protein. We constructed a hybrid protein containing EGFP fused to Im2. The gene encoding EGFP-Im2 was introduced in the arabinose-inducible pBADHisC vector just upstream of a sequence encoding a His_6 tag (pBAD-EGFP-Im2). The His_6-EGFP-Im2 protein was immunodetected with antisera directed against GFP or against ColE2-Im2 complex (Fig. 1A). As shown in Fig. 1A, His_6-EGFP had an apparent molecular mass of 35 kDa instead of 27 kDa for EGFP alone. In agreement with the apparent molecular mass of His_6-EGFP, the full fusion His_6-EGFP-Im2 had an apparent molecular mass of 48 kDa (Fig. 1A). The full-length fusion was stable, and there was no evidence for proteolytic cleavage. We showed by cell fractionation experiments that His_6-EGFP-Im2 was recovered in the cytoplasm and that no inclusion bodies could be detected in the membrane fraction, suggesting that the protein was correctly folded (Fig. 1B). From now, His_6-EGFP-Im2 will be noted EGFP-Im2.

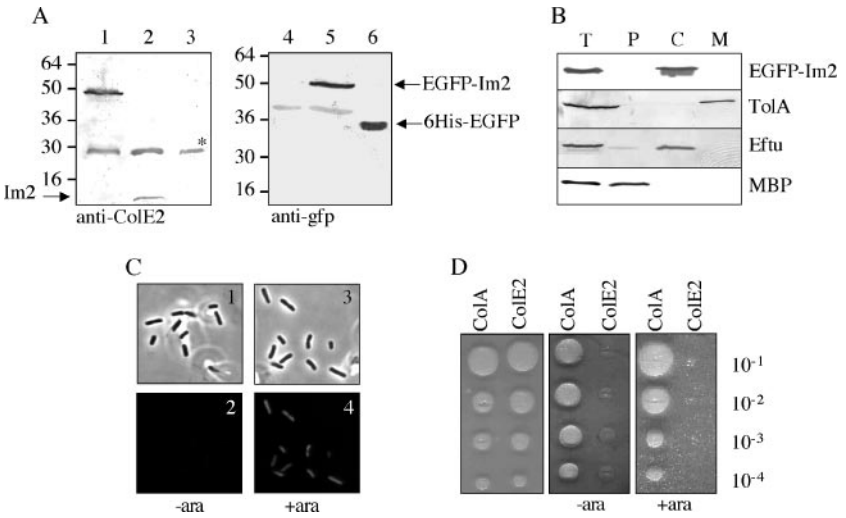

FIG. 1. Synthesis and in vivo activity of EGFP-Im2 hybrid protein. (A) Immunoblot analysis of EGFP-Im2 hybrid protein. Strain MC4100 cells containing plasmids encoding EGFP-Im2 (lanes 1 and 5), Im2 (lanes 2 and 4), and His_6-EGFP (lanes 3 and 6) were induced with 0.5 mg of arabinose/ml (lanes 1 and 5) or 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside) (lanes 2 to 4 and lane 6) for 30 min at 37°C. Cell extracts (A_{600} = 0.3) were immunoblotted, and the presence of various proteins was detected with antibodies directed against colicin E2 (lanes 1 to 3) and GFP (lanes 4 to 6). *, Contaminant protein recognized by antibodies directed against colicin E2. (B) Intracellular location of EGFP-Im2. Cells (T) producing EGFP-Im2 were induced with 0.5 mg of arabinose/ml for 30 min at 37°C and separated into cytoplasmic (C), periplasmic (P), and membrane (M) fractions as described in Materials and Methods. Fractions of 0.5 unit (A_{600}) were tested for the presence of EGFP-Im2 by immunoblotting with anti-GFP. The same fractions were tested for the presence of periplasmic maltose-binding protein, membrane-associated TolA, and cytoplasmic EF-Tu markers. (C) Expression and distribution of EGFP-Im2 fusion protein. MC4100 cells expressing (panels 3 and 4) or not (panels 1 and 2) EGFP-Im2 protein were visualized by fluorescence microscopy with an integration time of 3 s (panels 2 and 4) or by phase-contrast of the same field (panels 1 and 3). (D) Biological phenotype of EGFP-Im2 protein. Agar plates were overlaid with *E. coli* MC4100 cells expressing (middle and right panels, with [+] or without [−] 0.5 mg of arabinose/ml, respectively) or not (left panel) the EGFP-Im2 protein. Then, 1 μl of a serial dilution of either colicin A or colicin E2 was dropped onto each plate. The absence of zones of killing indicates biological resistance to ColE2 by the expressed EGFP-Im2 protein.

The two parts of EGFP-Im2 are functional. To verify that the EGFP part of EGFP-Im2 was functional in vivo, we examined expressing cells under a fluorescence microscope (Fig. 1C). Induction of EGFP-Im2 in *E. coli* resulted in the accumulation of fluorescence in the cell. According to cell fractionation assays, we concluded that the EGFP part of EGFP-Im2 was active in the cytoplasm of *E. coli* cells. The biological efficiency of cytoplasmic EGFP-Im2 to protect cells against colicin E2 was tested by using an agar plate assay. The level of sensitivity of wild-type MC4100 cells harboring the control plasmid or pBAD-EGFP-Im2 (with or without arabinose induction) was tested against serial dilutions of purified ColE2 and purified ColA. Figure 1D shows that MC4100 strain containing the control plasmid was sensitive to the bactericidal activity of purified colicins, whereas the same strain harboring pBAD-EGFP-Im2 was specifically resistant to ColE2, suggesting that a small amount of EGFP-Im2 was produced in the cells without induction. As expected, the induction of EGFP-Im2 in MC4100 resulted in complete resistance to ColE2. In all tested conditions MC4100 remained sensitive to ColA. Thus, the expression of the gene for the EGFP-Im2 protein conferred full biological immunity to ColE2. In conclusion, the two parts of EGFP-Im2 were fully functional.

Association of purified EGFP-Im2 and unbound ColE2. The results described above show that the produced EGFP-Im2 protein functions in vivo. Since our ultimate aim is to determine where and when the ColE2/EGFP-Im2 complex is dissociated, it was first essential to demonstrate that purified EGFP-Im2 could bind in a stoichiometric manner to uncomplexed ColE2, leading to its complete inactivation. The first stage of such a strategy involved the purification of EGFP-Im2 protein and unbound ColE2 protein. ColE2, unbound ColE2, and EGFP-Im2 were purified as described in Materials and Methods. The binding of purified EGFP-Im2 to unbound ColE2 was investigated in two separated experiments: gel filtration and inhibition of DNase activity.

Gel filtration analysis of purified EGFP-Im2 (37 kDa) and unbound ColE2 (62 kDa) in sodium phosphate buffer revealed two single well-defined A_{280} peaks that eluted at 13.40 and 12.85 ml, respectively (Fig. 2B). The elution profile of these two proteins corresponded to their relative differences in molecular mass. It should be noted that only 55 to 62% of the loaded proteins (at the amount of proteins used in these experiments) were recovered in the eluted fractions (82 μg instead of 150 μg for EGFP-Im2 and 155 μg instead of 250 μg for ColE2). Preincubation of stoichiometric amount of unbound ColE2 with EGFP-Im2 prior to loading onto column shifted the ColE2 A_{280} peak from 12.85 to 12.46 ml (Fig. 2B). This single peak accounted for more than 96% of the total peak area, and its elution was compared to that of known molecular mass markers (Fig. 2A). The result indicates that the ColE2/EGFP-Im2 complex was present in solution as a 106-kDa protein, which is a good agreement with the expected size for the complex. As described above, 66% of the total amount of protein loaded was recovered in the eluted fraction. Finally, this fraction loading onto sodium dodecyl sulfate-polyacrylamide

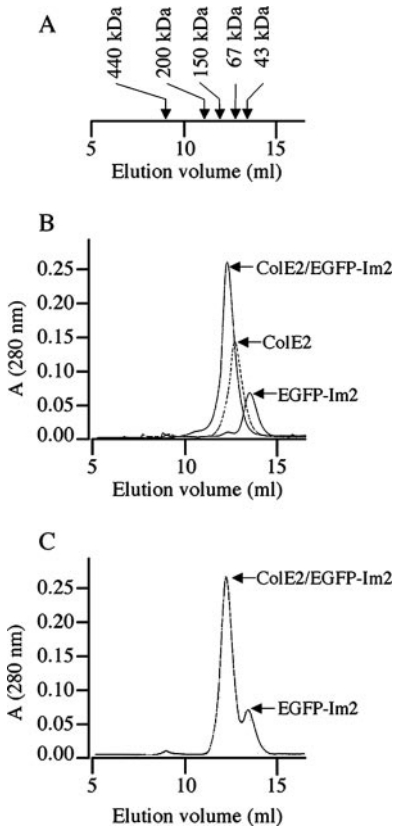

FIG. 2. Association of EGFP-Im2 with colicin E2 observed by gel filtration chromatography. The physical association of the two proteins was monitored by gel filtration chromatography as described in Materials and Methods. (A) The elution volumes of individual standard proteins are indicated: ferritin (440,000 Da), alcohol dehydrogenase (200,000 Da), β-amylase (150,000), bovine serum albumin (67,000 Da), and ovalbumin (43,000 Da). Elution of protein from a Superdex 200 column was recorded by tracing the A_{280} profile. (B) Elution profiles of EGFP-Im2 (150 μg), ColE2 (250 μg) and stoichiometric amount of ColE2 (250 μg) and EGFP-Im2 (150 μg) incubated together before they were applied to the column. The three profiles correspond to three independent experiments, and each individual peaks are annotated. (C) Elution profile of ColE2 (250 μg) preincubated with a twofold molar excess of EGFP-Im2 (300 μg).

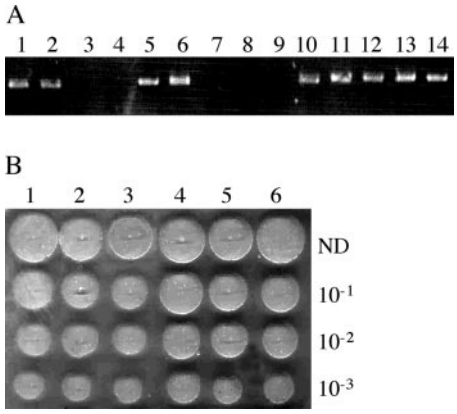

FIG. 3. Purified EGFP-Im2 inhibits the DNase activity of colicin E2. (A) Nuclease activity of ColE2 and its inhibition by EGFP-Im2. Ethidium bromide-stained agarose gel of electrophoresed target DNA (corresponding to 150 ng of linearized pUC18) after a 1-h incubation at 37°C with digestion buffer (lanes 1 and 14), purified EGFP-Im2 (0.43 μg, lane 2), increasing molar ratios of EGFP-Im2/ColE2 (0.3:1, 0.6:1, 1:1, and 1.3:1, respectively, lanes 3 to 6), refolded ColE2 (0.73 μg, lane 7), increasing molar ratios of Im2/ColE2 (0.3:1, 0.6:1, 1:1, and 1.3:1, respectively, lanes 8 to 11), native ColE2 complex (0.86 μg, lane 12), or refolded Im2 (0.12 μg, lane 13). (B) In vivo activity of ColE2/EGFP-Im2. Plate was overlaid with *E. coli* MC4100 cells. Portions (1 μl) of serial dilutions of free ColE2 (lane 1), reconstituted ColE2/EGFP-Im2 complex (lanes 2 to 3), reconstituted ColE2/Im2 complex (lanes 4 to 5), or native ColE2/Im2 complex (lane 6) were dropped onto plates. Large clear zones indicate bacterial cell death. Lane 1, unbound ColE2 (0.5 mg/ml); lanes 2 and 3, increasing molar ratios of EGFP-Im2/ColE2 (0.3:1 and 1.3:1, respectively); lanes 4 to 5, increasing molar ratios of Im2/ColE2 (0.3:1 and 1.3:1, respectively); lane 6, native ColE2/Im2 complex (0.5 mg/ml).

gel electrophoresis (12% acrylamide) contains both the colicin and EGFP-Im2 (data not shown). Incubation of a twofold molar excess of EGFP-Im2 to ColE2 prior to loading onto a column resulted in two A_{280} peaks that eluted at 12.85 ml and 13.40 ml, which corresponded to the elution profile of ColE2/EGFP-Im2 complex and free EGFP-Im2, respectively, suggesting that the complex is composed by an equimolar ratio of EGFP-Im2 and ColE2 (Fig. 2C).

Colicin E2 is known to be a nonspecific endonuclease (29). Refolded ColE2 was tested for DNase activity against linearized pUC18 plasmid DNA (Fig. 3A, lane 7); complete digestion was obtained after 1 h at pH 8.0 and 37°C. As controls, we showed that purified EGFP-Im2 and Im2 proteins did not possess any detectable DNase activity (Fig. 3A, lanes 2 and 13). To test whether EGFP-Im2 prevents the action of ColE2, both proteins were incubated at different molar ratios before the addition of the DNA substrate (Fig. 3A, lanes 3 to 6). We observed that the DNA substrate was only protected from digestion when the colicin was completely bound with EGFP-Im2 (1:1 stoichiometry). A similar result was obtained with purified Im2 protein added to unbound ColE2 (Fig. 3A, lanes 8 to 11) or with the native ColE2 complex (e.g., ColE2 with endogenous Im2, Fig. 3A, lane 12). Finally, in vivo activity of ColE2/EGFP-Im2 was tested by using an agar plate assay. As showed in Fig. 3B, reconstituted ColE2/EGFP-Im2 complex and ColE2/Im2 complex were as active as ColE2 alone or native ColE2 complex. This indicates that EGFP-Im2 associated with ColE2 did not prevent colicin import into sensitive cells.

Localization of immunity protein in the culture medium. To investigate whether EGFP-Im2 was released into the medium, supernatant and pellet fractions of broth cultures of *E. coli* MC4100 were collected 30 min after addition of ColE2/EGFP-Im2 complex at a multiplicity of 400 (number of complexes per cell). Portions (10 ml) of the cultures were centrifuged. Cells were collected, and supernatants were concentrated by ultrafiltration. The content of each fraction was analyzed by immunoblotting and the overlay method with antisera directed against GFP or ColE2 (see Materials and Methods). As showed in Fig. 4A, ColE2 was recovered in the cell fraction, whereas EGFP-Im2 was recovered in the supernatant, indicating that EGFP-Im2 dissociated from the bacteriocin complex. The same experiment was carried out with *E. coli btuB* strain. As expected, both ColE2 and EGFP-Im2 were recovered in the supernatant, confirming that ColE2 requires BtuB protein to bind cells and further indicating that the amount of bacteriocin

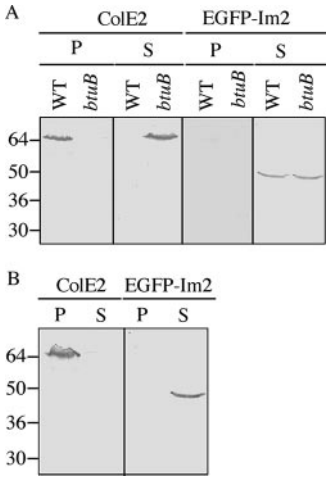

FIG. 4. Release of immunity protein from ColE2-treated cells. (A) Wild-type and *btuB* mutant strains incubated with ColE2/EGFP-Im2. The wild-type MC4100 strain and *btuB* mutant derivative were grown in LB medium at 37°C to an OD_{600} of 0.75, followed by incubation for 30 min with ColE2/EGFP-Im2 at a multiplicity of 400. Pellet (P) and supernatant (S) were separated by centrifugation, and the content of each fraction was analyzed by Western blotting with monoclonal antibodies directed against GFP (panel EGFP-Im2) or by the overlay method with purified ColA-Im2 (32), which specifically recognizes ColE2 (panel ColE2) (see Materials and Methods). Anti-mouse secondary antibodies were labeled with Alexa Fluor 488 dye (Molecular Probes). (B) Wild-type MC4100 strain incubated with free ColE2 or EGFP-Im2. Cells were prepared as described above but incubated with free ColE2 or EGFP-Im2 as indicated.

complex added to bacteria did not lead to nonspecific binding. As controls, we showed that free EGFP-Im2 incubated with MC4100 cells was recovered in the supernatant fraction, whereas unbound ColE2 incubated with MC4100 cells was recovered in the pellet fraction (Fig. 4B).

Further experiments at 37°C with MC4100 strain revealed that the release of EGFP-Im2 was a time-dependent process. Indeed, Fig. 5A shows that the amount of EGFP-Im2 detected in the cell fraction decreased regularly from 0 to 20 min after addition of the bacteriocin complex. During the same laps of time the amount of EGFP-Im2 detected in the supernatant fraction increased. In contrast, the amount of ColE2 detected in the cell fraction did not change with time (Fig. 5A). The same experiment performed at 4°C indicated that the immunity protein was not released in the supernatant even 60 min after the addition of the complex (Fig. 5B).

Release of the immunity protein required a functional receptor complex. From the result described above, it is hypothesized that the binding to BtuB is not sufficient to promote the release of the immunity protein from the bacteriocin complex. To cross the OM, the colicin N-terminal domain might recruit a porin (16). ColE2 uses preferentially OmpF as OM porin but can also use OmpC and PhoE (25). However, under standard growth laboratory conditions, OmpF and OmpC are the major porins of *E. coli* K-12. PhoE is only synthesized under phosphate limitation growth conditions (26). The in vivo activity of ColE2/Im2 tested using an agar plate assay showed that the complex remained fully active on the *ompF* mutant strain in contrast to colicin A, whereas it was completely inactive on the *ompF ompC* mutant strain (data not shown). This indicates

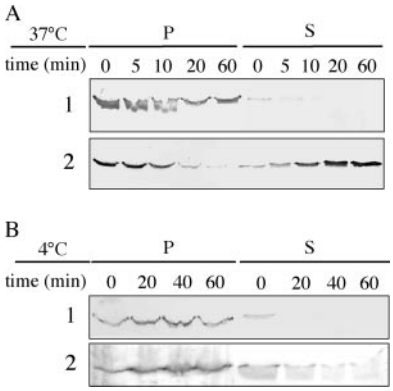

FIG. 5. Kinetics of immunity protein release. Wild-type strain MC4100 was grown in LB medium at 37°C to an OD_{600} of 0.75 and incubated with ColE2/EGFP-Im2 at a multiplicity of 400 at 37°C (A) or 4°C (B). At the indicated times, pellet (P) and supernatant (S) were separated by centrifugation, and the content of each fraction was analyzed by Western blotting with antibody directed against GFP (panel 2) or by the overlay method with purified ColA-Im2, which specifically recognizes ColE2 (panel 1).

that, as previously suggested, ColE2 can use either OmpF or OmpC to initiate its translocation through OM. As a control, we showed that colicins that do not use OmpF or OmpC in their mode of action were fully active (data not shown). In order to confirm that the binding of ColE2/EGFP-Im2 to BtuB is not sufficient to release the immunity protein, the complex was added at the multiplicity of 400 on wild-type, *ompF* mutant, or *ompF ompC* mutant bacteria. Figure 6A shows that EGFP-Im2 was not detected in the supernatant fraction when the bacteriocin complex was added to *ompF ompC* mutant bacteria but remained associated with the pellet fraction. In contrast, EGFP-Im2 was recovered in the supernatant fraction when the toxic complex was added to wild-type or *ompF* mutant bacteria, whereas ColE2 remained associated with the pellet fraction (Fig. 6A). These results demonstrate that the

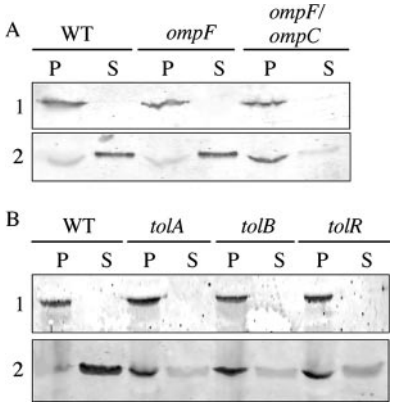

FIG. 6. Release of EGFP-Im2 required functional import machinery. (A) Wild-type MC4100 strain, the *ompF* mutant derivative, and the *ompF ompC* mutant strain SM1005 were incubated with ColE2/EGFP-Im2. Cells and samples (P, pellet; S, supernatant) were prepared as described in the legend to Fig. 4. (B) Wild-type C600 strain and *tolA*, *tolB*, and *tolR* mutant derivatives were incubated with ColE2/EGFP-Im2. Cells and samples (P, pellet; S, supernatant) were prepared as described in the legend to Fig. 4.

immunity protein is not released in the supernatant when the complex is bound to BtuB and further suggest that a subsequent step is required for the release of the immunity protein.

Release of immunity protein required a functional Tol complex. After binding to BtuB, and translocation through the OM, specific N-terminal sequences within E colicins must contact the Tol/Pal complex in the periplasm in order for the toxin to translocate into the bacterium. It has been proposed that the N-terminal domain of group A colicins once translocated across the OM near or through a porin interact with TolB. This interaction between colicins and TolB would then allow the colicins to penetrate further into the periplasm and to interact successively with the C terminus of TolA and finally with the TolR subunit near the inner membrane (18). In view of this version of the colicin journey across the cell envelope, we analyzed the function of the Tol proteins for the ColE2/Im2 dissociation. As in the above experiments, ColE2/EGFP-Im2 was added to wild-type or *tolA*, *tolB*, or *tolR* mutant strains. Pellet and supernatant fractions were collected 30 min after the addition of the complex and analyzed for the presence of either ColE2 or EGFP-Im2. As shown in Fig. 6B, EGFP-Im2 and ColE2 were recovered in the pellet fraction of *tol* cells. From these results, we conclude that the Tol proteins are absolutely required for the release of the immunity protein and further suggest that the ColE2 N-terminal domain must be fully translocated across the cell envelope to trigger the release of the immunity protein associated with the C-terminal domain located at the cell surface.

The immunity protein is not released from the periplasm. The results described above indicate that EGFP-Im2 was recovered in the supernatant 20 min after the addition of the bacteriocin complex to the cells and that this release required functional receptor and import machinery. To test whether the immunity protein is directly released from the OM or first translocated with the ColE2 C-terminal domain into the periplasm and then released into the medium, we performed cell fractionation experiments. Cells were recovered 5 or 20 min after addition of the bacteriocin complex and converted into periplasm, cytoplasm, and membrane fractions (see Materials and Methods). Figure 7A shows that the hybrid immunity protein associated with the cells, at 5 or 20 min after the addition of the bacteriocin complex, was exclusively recovered in the membrane fraction. No signal was detected in the periplasm fraction. However, to exclude the possibility that the hybrid immunity protein might be associated with the inner membrane or the inner face of the OM, we carried out protease accessibility experiments. Cells were first incubated with bacteriocin complex (5 or 20 min) and then treated or not treated with 200 μg of trypsin/ml. Cell and supernatant fractions were collected and analyzed with antiserum directed against GFP (Fig. 7B). We showed that after 5 min of preincubation with cells, EGFP-Im2 associated with the cell fraction was susceptible to trypsin, while a protein band of about 35 kDa appeared in the supernatant. This latter band is likely to represent the GFP fragment, which has a protease-resistant conformation (see Fig. 7C). At 20 min after addition of the bacteriocin complex to the cells, EGFP-Im2 was released into the supernatant but similarly digested by trypsin (Fig. 7B). As controls, we showed that the pattern of trypsin digestion without preincubation with cells of purified EGFP-Im2 or EGFP-Im2/ColE2 complex was identical (Fig. 7C). In sum, we demonstrate that EGFP-Im2 was released into the supernatant 20 min after the addition of ColE2/EGFP-Im2 to the cells and that during this period EGFP-Im2 remained accessible from the external medium.

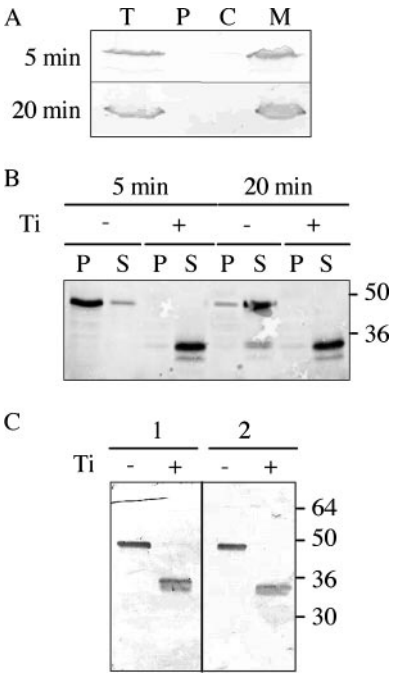

FIG. 7. Protease accessibility of EGFP-Im2. (A) Wild-type MC4100 strain was grown in LB medium at 37°C to an OD_{600} of 0.75 and incubated with ColE2/EGFP-Im2 at a multiplicity of 400 at 37°C. At the indicated times, the cells (T) were separated into cytoplasmic (C), periplasmic (P), and membrane (M) fractions as described in Materials and Methods. Fractions of 0.3 unit (A_{600}, sample at 5 min) or of 1 unit (A_{600}, sample at 20 min) were tested for the presence of EGFP-Im2 by immunoblotting with anti-GFP. (B) Wild-type MC4100 cells were prepared as described above and incubated with ColE2/EGFP-Im2 at a multiplicity of 400 at 37°C. At the indicated times trypsin (8.3 μM) was (+) or was not (−) added for 5 min at 37°C, and digestion was stopped by a molar excess of trypsin inhibitor. Pellet (P) and supernatant (S) were separated by centrifugation, and the content of each fraction was analyzed by Western blotting with antibody directed against GFP. (C) Purified EGFP-Im2 (100 nM, panel 1) and ColE2/EGFP-Im2 (100 nM, panel 2) were incubated with (+) or without (−) trypsin (8.3 μM) at 37°C for 5 min, and digestion was stopped by a molar excess of trypsin inhibitor. The presence of EGFP-Im2 was analyzed by Western blotting with antibody directed against GFP.

DISCUSSION

Since the first identification of DNase- and RNase-specific immunity proteins, it has been clear that the interaction between the toxin and its cognate immunity protein must be tight, because denaturation is needed to separate them (17). Indeed, several years later it was determined that immunity proteins bind to the C-terminal domain of enzymatic colicins with an affinity comparable to that of the avidin/biotin complex (37). However, enzymatic colicins have to dissociate themselves from immunity proteins to become active enzymes. In fact, immunity protein was released in the culture medium during

import of its cognate enzymatic colicin (20). However, the mechanistic bases underlying this dissociation remain unknown.

In the present study, we constructed a hybrid protein, EGFP-Im2 to facilitate the detection of Im2 in Western blot analyses. Despite its high molecular weight compared to Im2, the EGFP domain did not interfere with the CTE2 binding site on Im2. Indeed, bacteria producing EGFP-Im2 are efficiently and specifically protected against ColE2, and purified EGFP-Im2 interacts specifically with unbound ColE2 in a one-to-one molar ratio, resulting in complete inhibition of colicin DNase activity (Fig. 1 to 3). Moreover, the hybrid ColE2/EGFP-Im2 complex killed susceptible cells as efficiently as the wild-type complex or unbound ColE2, indicating that the EGFP part did not prevent binding to BtuB and translocation of the ColE2. This might be the result of the particular structure of colicins. Indeed, colicins have hairpin-like structures with a central receptor-binding domain (R) flanked by N-terminal translocation (T) and C-terminal cell killing (C) domains, the latter two in close proximity and separated from the R domain by a long coiled coil and immunity proteins of enzymatic colicins buried between the T and C domains (33, 39).

As previously described for the RNase cloacin DF13 (20), we showed in the present study that the immunity protein of the DNase ColE2 was released into the surrounding medium during import of the toxin. This dissociation was achieved 20 min after the addition of the ColE2/EGFP-Im2 complex to susceptible cells compared to 1 h for cloacin DF13. Our results suggest that enzymatic colicins reach their target at least 20 min after being in contact with cells. A similar value was found using an SOS promoter-*lux* fusion reporter assay to monitor DNA damage in colicin-treated cells (35). In contrast, the formation of membrane channels by pore-forming colicins, such as ColA, monitored in vivo by measuring the potassium efflux, occurs after a lag time of only 30 s at 37°C (13). As previously discussed, this is unlikely to be caused by differences in receptor binding and translocation, since identical helper proteins are involved for both colicins (35). This discrepancy might be explained by two other factors. First, in contrast to pore-forming colicins, enzymatic colicins are released as heterodimeric complexes with their cognate immunity protein, which must be removed during the translocation process; second, pore-forming colicins insert into the inner membrane to form channels, whereas nuclease colicins must cross the inner membrane to gain entry into the cytoplasm. The first hypothesis might be discarded because the times required for the full translocation of ColE9/Im9 complex and free ColE9 were identical (35). Here, we clearly showed that immunity protein was released in the culture medium approximately 20 min after the addition of the complex to the cells. Since the release occurs upon complex dissociation it is likely that before this time, the immunity protein was still in contact with the C-terminal domain of its cognate colicin. At the same time scale, the ColE9 C-terminal domain was detected in the cytoplasm of susceptible cells (35). Altogether, these results suggest that just after removal of immunity proteins, the C-terminal domain of nuclease colicins enter into the cytoplasm. Thus, it is unlikely that nuclease colicins take a significant time to cross the inner membrane. Another explanation would be that the immunity protein is imported with the C-terminal domain into the periplasm and released only in close proximity to the inner membrane. The C-terminal domain will thus begin its import through the inner membrane, while the immunity proteins will be secreted across the OM. In these conditions, the translocation of the C-terminal domain across the inner membrane would be the limiting step. However, in the present study we demonstrated that EGFP-Im2 was always accessible to exogenous protease, suggesting that this protein never entered into the cell. We cannot exclude that the EGFP domain of the hybrid immunity protein prevent immunity internalization, but in this case the release of the immunity protein should be faster.

The "colicin community" has proposed a model for the translocation of E-colicins and colicin A. Colicin import is initiated when the toxin binds to the BtuB receptor via its central domain (28). This first step concentrates the colicin on the membrane surface, changing the colicin diffusion from three to two dimensions (21). Through the extended coiled-coil structure, the colicin-BtuB complex may subsequently contact a second OM protein that may act as a translocator. This second OM protein is the OmpF porin. The N-terminal domain thus crosses the OM near or through OmpF and interacts with TolB, which is close to the OM due to its interaction with the peptidoglycan associated lipoprotein (Pal). Finally, the other Tol proteins would bring the N-terminal domain to the inner membrane that in some way open a pathway in the OM that allows entry the cytotoxic domain. Previous studies have suggested that upon binding to BtuB, ColA, and ColE3 immediately unfold to promote their movement into the cells (21, 12). Such an unfolding could be the driving force that dissociates the immunity-colicin complex. To test this hypothesis, ColE2/EGFP-Im2 was added to *ompF*/*ompC* cells. In this strain, the bacteriocin complex remains associated with its receptor BtuB but cannot initiate its translocation across the OM. Both ColE2 and EGFP-Im2 were recovered bound to bacteria, suggesting that binding to BtuB was not sufficient to release the immunity protein.

In an elegant study, Walker et al. (36) suggested using the thermodynamic consideration that the dissociation of Im3 from the ColE3-Im3 complex was dependent on the translocation of the N-terminal domain of ColE3 into the periplasm (36). Indeed, the crystal structure of the full-length ColE3-Im3 complex revealed that ImE3 interacts with both the catalytic and translocation domains of ColE3 (33). Thus, after the sequential interaction of the T-domain with OmpF and TolB, contacts between the T domain and Im3 are lost. As a consequence the interaction between the toxin and its cognate immunity protein is weakened, which will significantly increase the dissociation rate of the complex (36). As previously hypothesized, we showed that in the case of a DNase colicin, the engagement of the T-domain into or near the OmpF porin is not sufficient to release the immunity protein (36). In the *tolA* or *tolR* strain, whereas the ColE2 T-domain emerges into the periplasm and should interact with TolB but does not penetrate further into the envelope, the immunity protein remained associated with the colicin. This difference could reflect variations between the RNase and DNase immunity systems. Indeed, in the case of the DNase colicin E9 and its immunity protein, the affinity of the complex is not influenced by the presence of the T domain. Consequently, as previously described for ColE9 (37), bound immunity protein is not a prerequisite for cell attachment or translocation, since both free

colicin E2 and ColE/Im2 complex present a similar activity (Fig. 3B).

Recently, Cascales et al. (5) have indicated that the TolA protein was involved in transducing energy from the cytoplasmic membrane to the Pal lipoprotein and have hypothesized that this energy might be addressed via Pal lipoprotein to some other periplasmic or OM component (5). It is tempting to speculate that this energy might be involved in the ColE2/Im2 complex dissociation. However, until now, studies on the translocation of ionophoric colicins of group A have indicated that this process was energy independent (3). Nevertheless, this result does not exclude that the translocation of endonuclease colicins should be slightly different and required energy transduction via TolA.

In conclusion, in the present study we show that immunity proteins to DNase colicins, as has been previously described for RNase colicins (20), were released upon absorption of the colicin complex to the cell surface. We further demonstrate that release of Im2 required about 20 min and that during this time Im2 remains accessible from the external medium. Moreover, our results clearly demonstrate that the ColE2 T domain must interact with the Tol proteins and thus be fully translocated to promote dissociation of Im2 from the ColE2 C-domain. Thus, the release of immunity proteins is not an initial step in the nuclease colicin mode of action but one of the last.

ACKNOWLEDGMENTS

We gratefully acknowledge J. Sturgis for advice and discussions. We thank E. Cascales, P. Chames, and J. Sturgis for helpful comments on the manuscript.

This study was supported by a grant from the Centre National de la Recherche Scientifique and Vaincre la Mucoviscidose (no. II0531 to D.D. and R.L.).

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