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VirK Is a Periplasmic Protein Required for Efficient Secretion of Plasmid-Encoded Toxin from Enteroaggregative *Escherichia coli*

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Despite the autotransporter (AT) moniker, AT secretion appears to involve the function of periplasmic chaperones. We identified four periplasmic proteins that specifically bound to plasmid-encoded toxin (Pet), an AT produced by enteroaggregative *Escherichia coli* (EAEC). These proteins include the 17-kDa Skp chaperone and the 37-kDa VirK protein. We found that the *virK* gene is present in different *Enterobacteriaceae*. VirK bound to misfolded conformations of the Pet passenger domain, but it did not bind to the folded passenger domain or to the β domain of Pet. Assays with an EAECΔ*virK* mutant and its complemented version showed that, in the absence of VirK, Pet was not secreted but was instead retained in the periplasm as proteolytic fragments. In contrast, Pet was secreted from a Δ*skp* mutant. VirK was not required for the insertion of porin proteins into the outer membrane but assisted with insertion of the Pet β domain into the outer membrane. Loss of VirK function blocked the EAEC-mediated cytotoxic effect against HEp-2 cells. Thus, VirK facilitates the secretion of the AT Pet by maintaining the passenger domain in a conformation that both avoids periplasmic proteolysis and facilitates β-domain insertion into the outer membrane.

Secretion systems are used by many bacterial pathogens for the delivery of virulence factors to the extracellular space or directly into host cells. With their complex cell envelope, Gram-negative bacteria have evolved at least six general secretion systems to enable protein transfer across the inner membrane (IM), the periplasm, and the outer membrane (OM) (11). Some secreted proteins are exported across the IM and OM in a single step via the type I, type III, type IV, or type VI pathway. Other secreted proteins are first exported into the periplasmic space via the universal Sec-dependent or twin-arginine translocation pathway. These proteins are then translocated across the OM via type II, type V, or, less commonly, type I or type IV pathway (35). Among these pathways, one branch of the type V secretion system is notable for its apparent simplicity, the autotransporter (AT) mechanism. This pathway derives its name from the notion that AT proteins can, after Sec-dependent transfer to the periplasm, mediate their own translocation across the OM. The AT structure is composed of three major domains that contribute to its secretion; (i) a cleavable N-terminal signal sequence directs the protein to Sec-dependent passage into the periplasm, (ii) a passenger domain contains the effector function of the protein, and (iii) a C-terminal translocation unit forms a β-barrel pore in the OM that facilitates passenger domain export to the extracellular milieu (11). Although ATs constitute the largest family of secreted proteins in Gram-negative bacteria (26), the molecular mechanisms involved in AT secretion are not completely understood. Recent studies indicate that, contrary to the AT name, a variety of accessory factors are necessary for release of the AT passenger domain into the extracellular milieu.

AT secretion requires insertion of the AT β-barrel translocation unit into the OM. OM proteins (OMPs) are synthesized in the cytosol and translocated to the periplasm through the SecYEG translocon. OMPs pass through the translocon and enter the periplasm in an unfolded state before they form a β-barrel structure which inserts itself into the OM. In recent years, several studies have demonstrated that periplasmic proteins can serve as molecular chaperones in the assembly pathway of OMPs. For example, deletion of the genes encoding the 17-kDa Skp protein or the 48-kDa protein survival factor A (SurA) resulted in reduced concentrations of OMPs in the OM (17). Skp, SurA, and DegP are parts of a functional network that is vital for proper protein folding and degradation in the cell envelope (29). Skp and DegP function in one pathway, whereas SurA belongs to a separate, parallel pathway (29). In general, it seems that many periplasmic chaperones function in OMP biogenesis by preventing the misfolding and aggregation of a mostly unstructured protein folding intermediate. These chaperones and the β-barrel assembly machinery (BAM) system also appear to be involved in the OM insertion of the AT β-barrel translocation unit (12, 30, 31, 36).

Classical AT (monomeric) biogenesis likely involves an unfolded periplasmic intermediate of the passenger domain as well. An AT proprotein must pass through the narrow (~2-nm-diameter) pore of the Sec translocon en route to the periplasm, which suggests that the passenger domain enters the periplasm in an unfolded state (11). Once in the periplasm, the N-terminal signal sequence is proteolytically removed and the C-terminal domain forms a β-barrel pore which inserts itself into the OM. Translocation through an OM pore(s) then exposes the passenger domain to the extracellular milieu, where it may remain associated with the extracellular face of the OM or be cleaved and released into the medium. The nature of the OM translocation pore remains unknown, but it could involve the monomeric β domain, an oligo-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
HB101	Nonpathogenic strain, K-12/B hybrid	3
042	Wild-type EAEC strain from Peru	22
UT5600	Nonpathogenic strain, K-12 hybrid	16
MC4100	Nonpathogenic K-12 strain	32
MC4100Δ*skp*	Δ*skp* mutant	32
BL21/pLysDE3	Expression strain	33
EAECΔ*virK*::*kam*	Δ*virK* isogenic mutant	This work
EAECΔ*virK*::*kam*/pVirK	Δ*virK* isogenic mutant transformed with pVirK	This work
Plasmids		
pCEFN-1	3.9-kb fragment expressing Pet protein cloned into pSPORT1 (Amp^r)	9
pCEFN-2	Pet serine protease motif mutant	24
pRSET-A/*virK*	0.95-kb fragment of *virK* gene cloned into pRSET-A	This work
pKD46	Red recombinase expression	6
pKD4	Template for kanamycin resistance gene	6

meric β-domain complex, or the Omp85 pore (7, 38). In any case, it appears likely that the passenger domain would exit the periplasm and pass through the translocation pore in an unfolded or partially folded state. ATs engineered to express folded domains in the periplasmic space are not efficiently secreted through the OM translocation channel, providing further evidence that the secretion-competent passenger domain maintains a largely unfolded conformation while in the periplasm (14, 15). The structural state of the unfolded passenger domain would render it susceptible to proteolytic attack by periplasmic proteases, thus requiring a protective but transient interaction with periplasmic chaperones. Indeed, several studies have reported that the two parallel pathways of chaperone activity (i.e., Skp/DegP and SurA) for OMPs are also involved in passenger domain translocation across the OM (12, 14, 28, 30, 31, 36).

In this work, we identified a new component of the chaperone pathways involved with AT secretion. VirK was identified as a periplasmic protein that specifically binds to the passenger domain of plasmid-encoded toxin (Pet), a serine protease AT of the *Enterobacteriaceae* (SPATE) produced by enteroaggregative *Escherichia coli* (EAEC). VirK bound to misfolded but not folded conformations of the Pet passenger domain. VirK was required for the secretion of this SPATE protein from EAEC. In the absence of VirK, Pet degradation products accumulated in the periplasmic space. However, OM insertion of porin proteins was not affected by the loss of VirK. Our data suggest that VirK facilitates Pet secretion by protecting the unfolded AT passenger domain from proteolysis during transit through the periplasmic space.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. All strains were routinely grown aerobically at 37°C in Luria-Bertani (LB) broth. When necessary, the medium was supplemented with ampicillin (100 μg/ml), tetracycline (15 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (20 μg/ml).

Cellular fractionation. Bacterial strains were grown overnight at 37°C at 150 rpm in 50 ml of LB broth supplemented with the appropriate antibiotic. Supernatants were filtered through a 0.45-μm filter, and the proteins in the supernatant were precipitated with 10% (vol/vol) trichloroacetic acid (TCA) on ice for 1 h. The supernatants were then centrifuged at 14,000 ×*g* for 15 min, washed with acetone for 15 min, dried, and suspended in 100 μl of Tris-Laemmli buffer. A 10-μl portion of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (19) and visualized by Coomassie blue staining.

Periplasmic proteins were obtained from bacterial cultures in the logarithmic phase of growth. Bacterial pellets were resuspended in 10 ml of 30 mM Tris-HCl with 20% sucrose and 1 mM EDTA (pH 8.0), incubated for 10 min, and centrifuged at 8,000 ×*g* for 10 min. Bacterial pellets were resuspended in 500 μl of ice-cold 5 mM $MgSO_4$ and shaken for 10 min. The suspension was centrifuged at 8,000 ×*g* for 10 min at 4°C, and the supernatants containing the periplasmic fractions were collected and immediately analyzed by SDS-PAGE or stored at −20°C in Complete protease inhibitor cocktail (Boehringer Mannheim). The quality of the purified periplasmic fractions was tested by Western blot assay using anti-GroEL (kindly donated by Mario Cancino) and anti-β-lactamase (Chemicon, Temecula, CA) antibodies to detect GroEL (a cytoplasmic protein) and β-lactamase (a periplasmic protein), respectively.

OMs were obtained by the Sarkosyl extraction method as previously described (18). The bacterial cultures were grown as indicated above. Cultures were harvested by centrifugation at 7,000 ×*g* for 20 min at 4°C. The pellet was resuspended in 10 ml of 10 mM HEPES (pH 7.4), and the cells were lysed by sonication for 60 s (five times) in ice water using a Soniprep sonicator at 50% amplitude. Unbroken cells were removed by centrifugation at 2,500 ×*g* for 30 min at 4°C. The supernatant was centrifuged at 100,000 ×*g* for 1 h at 4°C. The resulting pellet was suspended in 10 ml of 1% Sarkosyl in 0.1 M Tris-HCl (pH 7.2) and incubated at room temperature for 30 min. The suspension was then centrifuged at 100,000 ×*g* for 1 h at 4°C. The pellet was suspended in 10 mM HEPES (pH 7.4). All samples were analyzed by SDS-PAGE.

Translocation unit purification and antibody production. OMPs from HB101(pCEFN1) were separated by preparative 12% SDS-PAGE and then stained with Coomassie blue. The protein band of the translocation unit was excised from the gel and electroeluted using an Electro-Eluter system (10 mA for 1 h) from Bio-Rad (Hercules, CA) and a protein elution buffer (25 mM Tris-base, 192 mM glycine, 0.1% SDS). SDS was removed from the protein samples by electrodialysis; the buffer in the electroelution chamber was replaced with fresh SDS-free elution buffer. To remove glycine from the buffer, the samples were dialyzed against phosphate-buffered saline (PBS) for 12 h. The protein was used in overlay experiments and to produce antibodies against the translocation unit.

Antibodies against the translocation unit were elicited by using the pure protein obtained from electroelution as described above and injecting the protein into female BALB/c mice. Antibody response and specificity were determined by immunoblotting, and the antisera were diluted according to their sensitivity.

SDS-PAGE and Western blotting. Periplasmic fractions and supernatant proteins from bacterial cells were quantified by the micro-Bradford method (4) and resolved by SDS-PAGE with 12% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes for Western blot analysis (34). Immunoblotting was performed using rabbit primary antibodies against Pet and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Zymed, Grand Island, NY) as indicated by the manufacturer. HRP was detected with the ECL reagent from Amersham (Piscataway, NJ).

Coimmunoprecipitation assays. Periplasmic proteins (800 μg) obtained from bacterial cultures expressing Pet (pCEFN1), the PetS260I mutant protein (pCEFN2), or Pet without the β-barrel translocation unit (pJPN205) were used for immunoprecipitation assays. A 5-μg sample of a polyclonal anti-Pet antibody and protein A-agarose suspension (Roche Diagnostics, Mannheim, Germany) was incubated with periplasmic proteins for 3 h at 4°C to precipitate the protein-antibody complex. The beads were washed three times with radioimmunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl,

0.02% SDS) and resuspended in loading buffer before the immunocomplexes were resolved by SDS-PAGE.

Overlay assay. Overlay assays were performed as previously reported (5). Periplasmic proteins (30 μg) were separated by SDS-PAGE with 12% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (Bio-Rad), which were blocked overnight at 4°C in blocking buffer (150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 [pH 7.3], 2 mM $CaCl_2$, 5% nonfat dry milk). The membranes were then incubated for 1 h in binding buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 2 mM $CaCl_2$, 5% bovine serum albumin) with either a 5-μg/ml concentration of PetS260I or the β-barrel translocation unit. The membranes were washed and then incubated for 1 h in blocking buffer with rabbit polyclonal anti-Pet antibodies (1:500 dilution) or mouse polyclonal anti-β-barrel-translocation-unit antibodies (1:500 dilution). Following another wash step, the membranes were incubated for 1 h in blocking buffer with an HRP-conjugated goat anti-rabbit IgG antibody or an HRP-conjugated goat anti-mouse IgG antibody (Zymed) as indicated by the manufacturer. HRP was detected with the ECL reagent from Amersham.

Pulldown assay. The periplasmic proteins (3 mg) from *E. coli* HB101 or UT5600 were incubated with PetS260I (60 μg) or the Pet β-barrel translocation unit (60 μg) for 3 h at 4°C. Anti-Pet or anti-translocation-unit antibodies were then added to the samples, followed by a protein A-agarose suspension (Roche Diagnostics, Mannheim, Germany). After another 3 h at 4°C, the complexes were collected by centrifugation and the supernatant was removed. The pellet was washed five times with RIPA buffer and resuspended in loading buffer before the immunocomplexes were resolved by SDS-PAGE.

Protein identification by MALDI-TOF. Candidate proteins isolated by pulldown assay were resolved by SDS-PAGE and stained with Coomassie blue. Excised gel slices were destained overnight (50% methanol, 5% acetic acid). After being washed with acetonitrile, proteins in the gel slices were reduced and alkylated with 10 mM dithiothreitol and 50 mM iodoacetamide, respectively. The gel slices were then treated with 50 mM ammonium bicarbonate and washed with acetonitrile. Protein was extracted from the gel slice using 5% formic acid and 50% acetonitrile solution. Enzymatic digestion of the extracted protein was performed with trypsin, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis was performed with a Voyager-DE PRO mass spectrometer. Samples were processed in reflectron mode and calibrated externally with a mixture of known peptide standards in a molecular mass range of 900 to 1,600 Da (Applied Biosystems, Carlsbad, CA). Identification of proteins was performed using ALDENTE (http://web.expasy.org/cgi-bin/aldente/help.pl?intro.html) and MASCOT databases. Proteins with the highest score, higher statistical significance ($P \leq 0.05$), a minimum of missed cleavages, a minimum of delta parts per million between the molecular masses of the experimental peptides and those of the corresponding theoretical peptides, a theoretical pI/molecular weight (MW) ratio close to the experimental pI/MW ratio, and more than 20% coverage were retained.

Construction of a *virK* mutant. To generate the isogenic *virK* mutant of EAEC, the *virK* gene was interrupted with a gene encoding kanamycin resistance by use of the lambda red recombinase system (6). The kanamycin resistance gene was amplified from pKD4 by PCR with primers *virK*-FRT forward (5′-ATG TTT TCT ATA AGT AAC TTA TCA TTT ATC GGT TTC CTT AAA AGG ATT GTT GTG TAG GCT GGA GCT GCT T-3′) and reverse (5′-TTT ACC AAA ATT ATC ATT ACT GTT TTA TTC AGA GGA TAC ATC AAA TTT TAC ATA TGA ATA TCC TCC TTA G-3′). The product was treated with DpnI and introduced into EAEC carrying pKD46. Colonies containing the *virK*::Km interrupted gene (referred to as EAECΔ*virK*) were then selected as previously described (6).

Expression and purification of VirK. The *virK* gene (GenBank accession no. AF134403) was amplified from plasmid DNA by PCR using the following primers: forward, 5′-GGA TCC ATG TTT TCT ATA AGT AAC TTATC ATT-3′; reverse, 5′-CTG CAG ATT TGA ATT TTG ATG TTT TGA GTG T ACA-3′ (BamHI and PstI restriction sites are underlined). The product was cloned into the PGEM-T Easy vector system kit as recommended by the manufacturer (Promega Corporation, Madison, WI). Subsequently, the insert was obtained through digestion with restriction enzymes BamHI and PstI. The insert was purified from an agarose gel with the QIAquick gel extraction kit and DNA purification system according to the technique established by the manufacturer (Qiagen, Valencia, CA). The pRSET-A expression vector was used to subclone the *virK* gene in order to get pRSET-A-pVirK-His, after which *E. coli* BL21/pLysDE3 was transformed with this construct. The construct was verified by DNA sequencing (ABI Prism 310 automated sequencer; Perkin-Elmer, Applied Biosystems), and overexpression of the fusion protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA) to the transformed bacterial culture. VirK-His_6 was purified by Ni-nitrilotriacetic acid (NTA) agarose affinity chromatography in accordance with the manufacturer's instructions (Qiagen). Protein concentration was determined by the Bradford assay, and purity was assessed by SDS-PAGE with 12% polyacrylamide gels.

Cell culture. The human cell line HEp-2 (ATCC CCL-23) was grown in minimum essential medium (MEM), supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, penicillin, streptomycin (Invitrogen), and 10% fetal bovine serum (PAA Cell Culture Co., Piscataway, NJ). Cells were incubated at 37°C with 5% CO_2. Cells were routinely harvested with 10 mM EDTA and 0.25% trypsin (Invitrogen) in PBS (pH 7.4), resuspended in the supplemented MEM, and incubated at 37°C in a humidified atmosphere of 5% CO_2. For all experiments, cells were used only during five consecutive passages.

Immunofluorescence microscopy. After bacterial interaction, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, washed, and permeabilized when required by the addition of 0.1% Triton X-100 in PBS. The actin cytoskeleton was detected by staining with 0.05 mg/ml tetramethyl rhodamine isothiocyanate-phalloidin. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and covered with glass coverslips. Preparations were analyzed and registered using a Leica TCS SP2 confocal microscope and prepared digitally with ImageJ Software (NIH).

Infection model. HEp-2 cells adjusted to 5×10^4 per well in LabTek slides (VWR, Radnor, PA) were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. The medium was then replaced with fresh Dulbecco's modified Eagle medium (DMEM; Invitrogen) lacking fetal bovine serum and antibiotics but with 1% tryptone, which enhances the expression and secretion of Pet from EAEC grown in DMEM (1). EAEC strain 042 was grown overnight at 37°C in LB broth. Kanamycin and/or ampicillin and 1 mM IPTG were added when required. EAEC bacterial cultures used to inoculate fresh DMEM–1% tryptone medium (multiplicity of infection, 20) were incubated with cells for 2 h.

RESULTS

Pet binds to periplasmic proteins. To determine whether Pet associates with periplasmic proteins during secretion, we performed coimmunoprecipitation assays using polyclonal antibodies against the passenger domain of Pet and the periplasmic fraction of Pet-expressing *E. coli*. Full-length, unprocessed Pet is a 140-kDa protein. Cleavage of the unusually long signal sequence generates a 134-kDa intermediate consisting of the passenger domain and the β-barrel translocation domain. Proteolytic nicking of this intermediate on the extracellular face of the OM releases the mature passenger domain into the medium as a 104-kDa toxin, while the β-barrel translocation domain remains embedded in the OM as a 30-kDa protein (23). Western blot analysis confirmed the purity of our periplasmic fraction, which contained β-lactamase but lacked cytoplasmic protein GroEL and OM protein OmpA (Fig. 1A).

AT translocation is very fast (15, 20), so we utilized two strategies to capture the transient interaction between Pet and potential chaperones. First, coimmunoprecipitation assays were per-

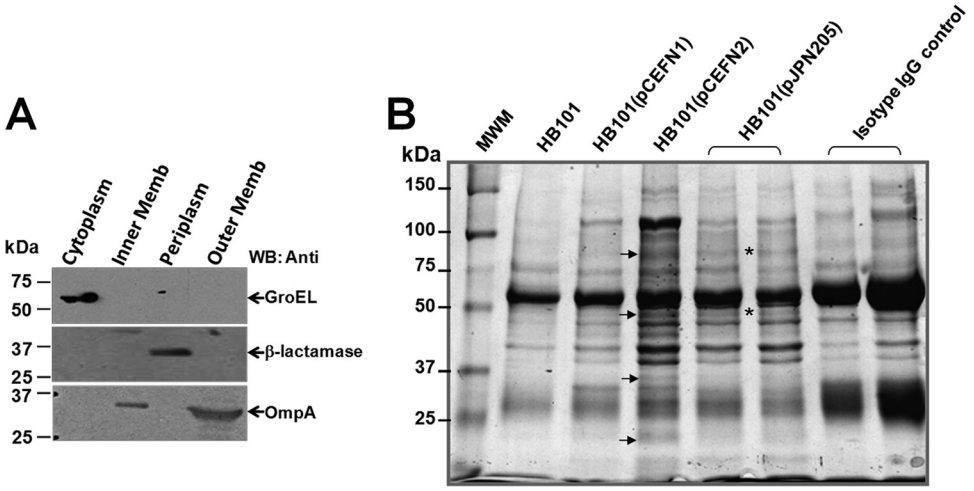

FIG 1 Pet interacts with specific periplasmic proteins from *E. coli* HB101 during secretion. (A) Bacterial strains grown overnight at 37°C at 150 rpm in 50 ml of LB broth were fractionated as described in Materials and Methods. Cytoplasmic, IM, periplasmic, and OM fractions were analyzed by Western blot (WB) assay using as markers antibodies against GroEL, β-lactamase, and OmpA. (B) The periplasmic fraction of *E. coli* HB101 was immunoprecipitated with an anti-Pet polyclonal antibody. Experiments were performed with an untransformed laboratory strain that does not express Pet (HB101), a strain transformed with a plasmid encoding wild-type Pet (pCEFN1), a strain transformed with a plasmid encoding the inactive mutant protein PetS260I (pCEFN2), and a strain transformed with a plasmid encoding the truncated mutant Pet protein that lacks the β-barrel translocation unit (pJPN205). An additional negative control involved coimmunoprecipitation of the periplasmic fraction from HB101(pCEFN1) with an isotype IgG control antibody. All immunocomplexes were resolved by SDS-PAGE and visualized by Coomassie blue staining. Arrows indicate the proteins (20, 37 50, and 80 kDa) that specifically interacted with PetS260I; asterisks denote the 50- and 80-kDa proteins that also bound to the truncated mutant Pet protein. MWM, MW markers.

formed with Pet expressed in *E. coli* laboratory strain HB101. Previous work has shown that HB101 secretes both folded and misfolded variants of Pet, whereas only correctly folded Pet is recovered from the culture supernatant of EAEC (25). Because HB101 produces a substantial pool of misfolded toxin, the transient interactions between Pet and periplasmic chaperones would likely be elevated in this strain. Second, coimmunoprecipitation assays were also performed with two Pet mutants that were expected to exhibit altered rates of secretion: a mutant form of Pet with a change in the serine protease motif (PetS260I) and a truncated mutant form lacking a portion of the passenger domain as well as the entire β-barrel translocation domain (9). PetS260I is secreted at low concentrations compared to native Pet (24), while the truncated mutant form would not be secreted at all but would instead be retained in the periplasm (9).

Immune complexes for wild-type Pet (pCEFN1), PetS260I (pCEFN2), and truncated Pet (pJPN205) were analyzed by SDS-PAGE (Fig. 1B). Four periplasmic proteins with apparent molecular masses of 20, 37, 50, and 80 kDa were coimmunoprecipitated with PetS260I (HB101/pCEFN2). These proteins were not immunoisolated in the absence of Pet expression (HB101) or with an irrelevant isotype IgG antibody control, thus demonstrating the specificity of Pet-protein interaction. No clear bands were immunoisolated with wild-type Pet [HB101(pCEFN1)], which indicated that delayed secretion of Pet was necessary to capture its interactions with these periplasmic proteins. Similar strategies to stall the translocation event with mutant toxins have been used to identify AT interactions with various periplasmic proteins (12, 14, 31). Interestingly, only the 50- and 80-kDa proteins appear to be immunoisolated with the truncated variant of Pet (HB101/pJPN205). This could suggest that the 20- and 37-kDa proteins bound to motifs in the C-terminal region of the passenger domain and/or the β-barrel translocation domain (i.e., the regions missing from the truncated mutant).

To further demonstrate the specificity of Pet binding to the periplasmic proteins identified by coimmunoprecipitation, we performed an overlay assay that has previously been used to identify protein-protein interactions (2, 5, 10) (Fig. 2). Periplasmic fractions were generated from *E. coli* HB101 and UT5600, a strain which expresses high levels of periplasmic proteins but lacks the OmpT and OmpP proteases (16). The periplasmic proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Separate membranes were then incubated with either purified PetS260I or the purified β-barrel translocation unit of Pet. *E. coli* HB101 was used as the source of PetS260I, thereby providing the assay with a mixed pool of both folded toxin and misfolded toxin. Protein interactions were detected by Western blot assay using antibodies against Pet or the translocation unit. PetS260I bound to 37- and 50-kDa proteins (Fig. 2A), whereas the β-barrel translocation unit interacted with the 20-kDa protein (Fig. 2C). For both PetS260I and the β-barrel translocation unit, interactions were more apparent in UT5600 than in HB101. To identify nonspecific interactions between the antibodies and periplasmic proteins from *E. coli*, we performed a Western blot assay using anti-Pet and anti-translocation-unit antibodies in the absence of an overlay with PetS260I (Fig. 2B) or the β-barrel translocation unit (Fig. 2D). The antibodies cross-reacted with some periplasmic proteins of *E. coli* but not with the 20-, 37-, and 50-kDa proteins specifically found in the overlay assays.

Slightly different results were obtained from the coimmunoprecipitation assay and the overlay assay, but this reflected the different methodologies of the two techniques. In the coimmunoprecipitation assay, proteins that do not directly bind to Pet could still be isolated as part of a toxin-binding complex. In contrast, the overlay assay resolves the periplasmic fraction as individual proteins before exposure to Pet. Detection of the 20-kDa and 80-kDa proteins in the coimmunoprecipitation assays of Fig. 1 but not in the overlay assay of Fig. 2A accordingly suggested that these pro-

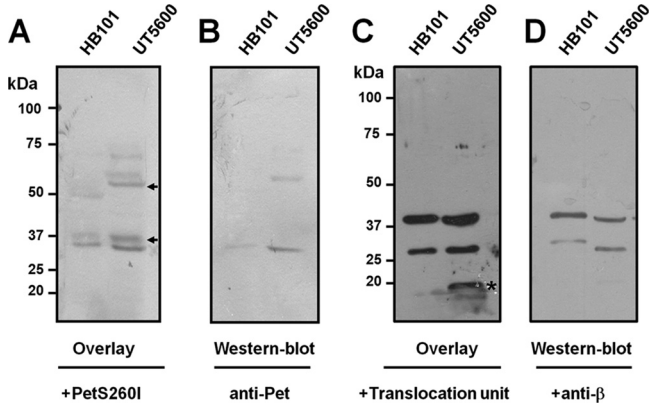

FIG 2 Differential interactions between *E. coli* periplasmic proteins and either the Pet passenger domain or the Pet β-barrel translocation unit. Overlay assays of Pet (A) or the β-barrel translocation unit of Pet (C) and periplasmic proteins are shown. The periplasmic proteins of *E. coli* HB101 and UT5600 were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with purified PetS260I (A) or the β-barrel translocation unit of Pet (C). To detect specific interactions between Pet or the β-barrel translocation unit of Pet and periplasmic proteins, a primary rabbit anti-Pet antibody or a primary mouse anti-translocation-unit antibody was used, followed by an HRP-conjugated anti-rabbit IgG secondary antibody or an HRP-conjugated anti-mouse IgG secondary antibody, respectively. (B and D) Western blot assay to confirm the specific interaction between periplasmic protein and either Pet or the β-barrel translocation unit. The periplasmic fractions of *E. coli*, as indicated in panels A and C, were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The nonspecific interaction between the periplasmic proteins and the primary antibodies was visualized by using a rabbit anti-Pet or a mouse anti-translocation-unit primary antibody, followed by an HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody, respectively. The arrows in panel A indicate the specific interaction between Pet and two periplasmic proteins with apparent molecular masses of 37 and 50 kDa, and the asterisk in panel C indicates the specific interaction between the β-barrel translocation unit and one periplasmic protein with an apparent molecular mass of 20 kDa.

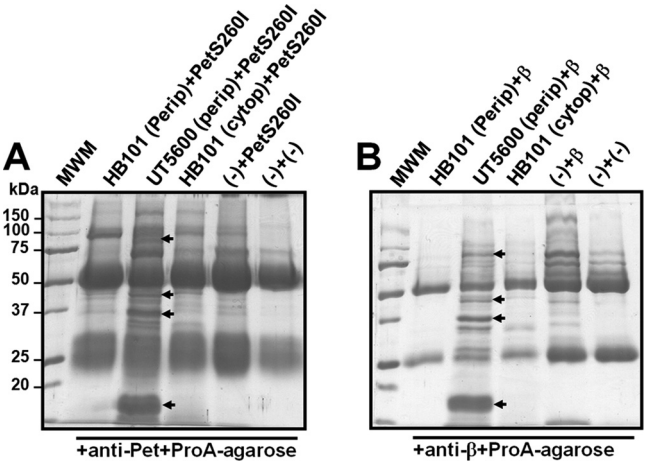

FIG 3 Pet and its translocation unit interact with periplasmic proteins from *E. coli* in pulldown assays. PetS260I (A) and the Pet β-barrel translocation unit (B) were incubated with periplasmic (perip) proteins from *E. coli* HB101, periplasmic proteins from *E. coli* UT5600, or cytoplasmic (cytop) proteins from *E. coli* HB101 or without *E. coli* proteins. The mixtures were subjected to immunoprecipitation with anti-Pet or anti-translocation-unit antibodies, and the immunocomplexes were separated by SDS-PAGE. An additional control resolved the antibodies in the absence of both toxin and *E. coli* proteins. Arrows indicate proteins that specifically bound to Pet and its translocation unit. MWM, MW markers.

teins interact with the Pet passenger domain only indirectly, as part of a toxin-binding complex. However, results from the β-domain overlay assay (Fig. 2C) indicate that the 20-kDa protein can bind directly to the Pet β domain.

It should also be noted that, as assessed by Western blot analysis, none of the β domain was detected in the periplasmic fractions from the Pet-expressing *E. coli* strain used in Fig. 1 (data not shown). This indicated that the β domain was efficiently inserted in the OM, with some of the passenger domain facing the periplasm and released into that compartment. Ruiz-Perez et al. (30) have likewise reported the presence of the mature passenger domain in their periplasmic fractions.

To further verify the binding between Pet and its four interacting periplasmic proteins, we performed pulldown assays using the periplasmic fractions from *E. coli* HB101 or UT5600 (Fig. 3). These fractions were incubated with either purified PetS260I or the purified β-barrel translocation unit. As with the overlay assay, PetS260I was obtained from *E. coli* HB101 culture supernatant. Interacting proteins were isolated by immunoprecipitation with anti-Pet or anti-translocation-unit antibodies. Analysis of the complexes by SDS-PAGE confirmed the interaction of Pet (Fig. 3A) and the translocation unit (Fig. 3B) with proteins of 20, 37, 50, and 80 kDa. The interactions were more evident with periplasmic fractions from UT5600 than with those from HB101, which was consistent with the results in Fig. 2 and was probably due to the OM protease defects in UT5600. All four interacting proteins were detected with pulldown assays using either PetS260I or the β-barrel translocation unit, which was distinct from the more limited pattern of interactions detected by our overlay assay (Fig. 2). These experimental differences again suggested the possible formation of a protein complex that could be isolated/detected with a pulldown assay but not with an overlay assay. Binding in the pulldown assay was specific, since similar pulldown experiments using cytoplasmic proteins instead of periplasmic proteins gave negative results. Control experiments in which the pulldown assay was performed in the absence of periplasmic proteins, in the absence of PetS260I, or in the absence of the β-barrel translocation unit further confirmed the specificity of the interactions between Pet and the four immunoisolated proteins (Fig. 3).

Three separate methods of demonstrating protein-protein interactions (coimmunoprecipitation, overlay, and pulldown) each detected the specific interaction between Pet and periplasmic proteins with apparent molecular masses of 20, 37, 50, and 80 kDa. These results showed that Pet interacts with proteins from the periplasmic space during secretion. The use of varied methods to detect protein-protein interactions further allowed us to roughly determine the nature of Pet binding to these periplasmic proteins. Our collective data indicate that the 20-kDa and 80-kDa proteins are recruited to the Pet passenger domain through interactions with the other identified periplasmic proteins: these 20-kDa and 80-kDa proteins did not bind directly to the Pet passenger domain (Fig. 2) but were isolated with other periplasmic proteins in coimmunoprecipitation (Fig. 1) and pulldown (Fig. 3) assays focused on the Pet passenger domain. Furthermore, the 20-kDa protein bound directly to the Pet β-barrel translocation domain, as determined by overlay assay (Fig. 2). This interaction was sufficient to recruit the other three periplasmic proteins which did not directly bind to the translocation domain (Fig. 2) but were isolated from

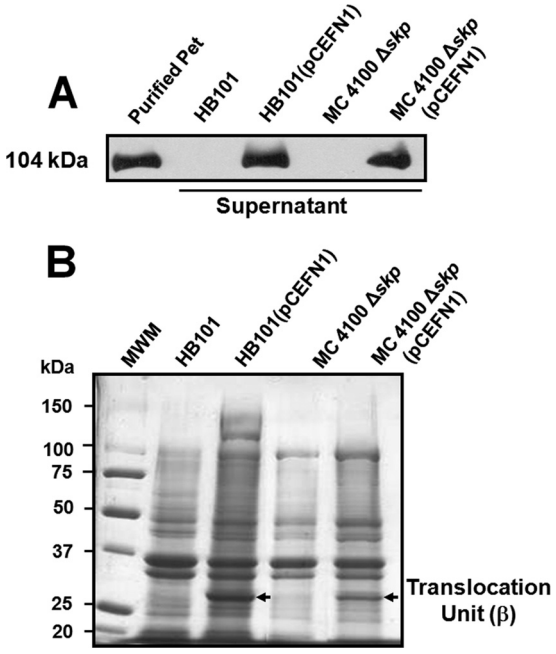

FIG 4 Secretion and OM insertion of Pet by *E. coli* MC4100Δ*skp*. (A) Supernatants were collected from overnight cultures of untransformed *E. coli* strains that did not express Pet (HB101 and MC4100Δ*skp*) or from the same *E. coli* strains transformed with a plasmid encoding wild-type Pet (pCEFN1). Proteins in the supernatants were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The secreted pool of Pet was detected by Western blot analysis using a rabbit anti-Pet polyclonal primary antibody and an HRP-conjugated anti-rabbit IgG secondary antibody. Supernatants were obtained from bacterial cultures (optical density at 600 nm of 1.0) under the same conditions for each strain and then precipitated by using 20% TCA. (B) OM proteins from the strains used in panel A were separated by SDS-PAGE and visualized by Coomassie blue staining. Arrows denote the β-barrel translocation unit of Pet which was embedded in the OM. MWM, MW markers.

the pulldown assay (Fig. 3). All four identified proteins thus appear to form a toxin-binding complex, with two proteins directly recognizing the Pet passenger domain and one protein directly recognizing the Pet β-barrel translocation domain.

Identification of Skp and VirK as Pet-binding proteins. To identify the proteins that interacted with Pet in our pulldown assay, we performed MALDI-TOF mass spectrometry. Peptide analysis was performed with the MASCOT software from Matrix Science. The 50- and 80-kDa proteins could not be identified, due to the low concentrations of recovered proteins and/or contamination with other proteins. However, we identified the protein with an apparent molecular mass of 20 kDa as Skp, a 17-kDa chaperone that has affinity for phospholipids, lipopolysaccharides, and OM proteins (8). Experiments with a mutant strain of *E. coli* lacking Skp (MC4100Δ*skp*) indicated that this chaperone is not required for the secretion of Pet: the passenger domain and the translocation unit were detected in the extracellular medium and in the OM fraction, respectively, of MC4100Δ*skp* (Fig. 4). The 37-kDa protein was identified as VirK. We found that the *virK* gene is present in different members of the family *Enterobacteriaceae* such as *E. coli*, *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Klebsiella* spp., *Vibrio* spp., and *Erwinia* spp. Furthermore, EAEC contains both a chromosomal and a plasmid-borne copy of *virK*. Originally identified in *Shigella flexneri*, VirK was thought to be important for posttranscriptional expression of IcsA, a non-SPATE AT (21). However, the role of VirK in IcsA biogenesis was not clearly determined.

VirK exhibits a specific interaction with the denatured or misfolded Pet passenger domain. To further examine the role of VirK in Pet biogenesis, we cloned *virK* from genomic EAEC DNA. The gene was placed in plasmid pRSET-A to generate a VirK-His_6 fusion construct with an N-terminal epitope tag. As expected, VirK-His_6 was located mainly in the periplasm of *E. coli* BL21/pLysDE3 transformed with pRSETA-pVirK-His (Fig. 5A), as detected in wild-type EAEC (data not shown). Western blot analyses confirmed the purity of our periplasmic fraction: it lacked GroEL but contained β-lactamase, which was absent from the IM, OM, and cytoplasmic fractions (Fig. 5A). Using cell lysates from *E. coli* BL21/pLysDE3 transformed with pRSETA-VirK-His, the recombinant Virk-His_6 protein was purified by affinity chromatography with agarose-Ni^{2+} columns. Virk-His_6 eluted in fractions 2 and 3 (Fig. 5B), which were subsequently used for studies involving pulldown assays (Fig. 5C and D).

For pulldown experiments, purified VirK-His_6 was mixed with purified Pet, purified and denatured Pet (boiled for 10 min), the purified β-barrel translocation unit, or the purified and denatured β-barrel translocation unit. As controls, the Pet passenger domain or the β domain was mixed with fraction 2 eluted from BL21/pLysDE3 transformed with the empty pRSETA vector. Proteins interacting with VirK were coimmunoprecipitated with an anti-His antibody and resolved by SDS-PAGE. VirK clearly interacted with denatured Pet but not with native Pet, the native β domain, or the denatured β domain (Fig. 5C). This result suggested that VirK interacts preferentially with the misfolded conformations of Pet. SDS-PAGE of supernatants recovered after the coimmunoprecipitation visualized the proteins that did not bind to VirK and confirmed that the β-domain samples were unable to interact with VirK-His_6 (Fig. 5D).

VirK is necessary for Pet secretion but not for porin insertion into the OM. Since VirK was identified as a Pet-binding protein, we examined whether it is involved in Pet secretion from EAEC. Both the chromosomal and plasmid-borne copies of *virK* were deleted from EAEC as described in Materials and Methods. To analyze the secretion of Pet from EAECΔ*virK*, we performed Western blot assays of culture supernatants using anti-Pet antibodies (Fig. 6A). Pet was not secreted from EAECΔ*virK*, which contrasted with its efficient secretion from the isogenic wild-type strain. Complementation of EAECΔ*virK* with a plasmid-borne copy of the *virK* gene (EAECΔ*virK*/pVirK) restored the secretion of Pet (Fig. 6A). These data suggested that VirK expression is necessary for the secretion of AT protein Pet.

To determine if VirK functions as a general chaperone, we monitored the insertion of *E. coli* porins into the OM of EAEC, EAECΔ*virK*, and EAECΔ*virK*/pVirK (Fig. 6B). Coomassie staining of OMPs separated by SDS-PAGE showed that the efficiency of porin insertion into the OM was similar for all three strains. However, EAECΔ*virK* was unable to properly insert the Pet β-domain into the OM. Thus, the Pet passenger domain secretion defect in EAECΔ*virK* did not result from a global secretion deficiency. As the loss of VirK function did not impair the OM insertion of porins, VirK apparently impacts the biogenesis of a subset of secreted proteins such as the AT protein Pet. The proper OM localization and function of omptin IcsP in *virK*::Tn*10* shigella mu-

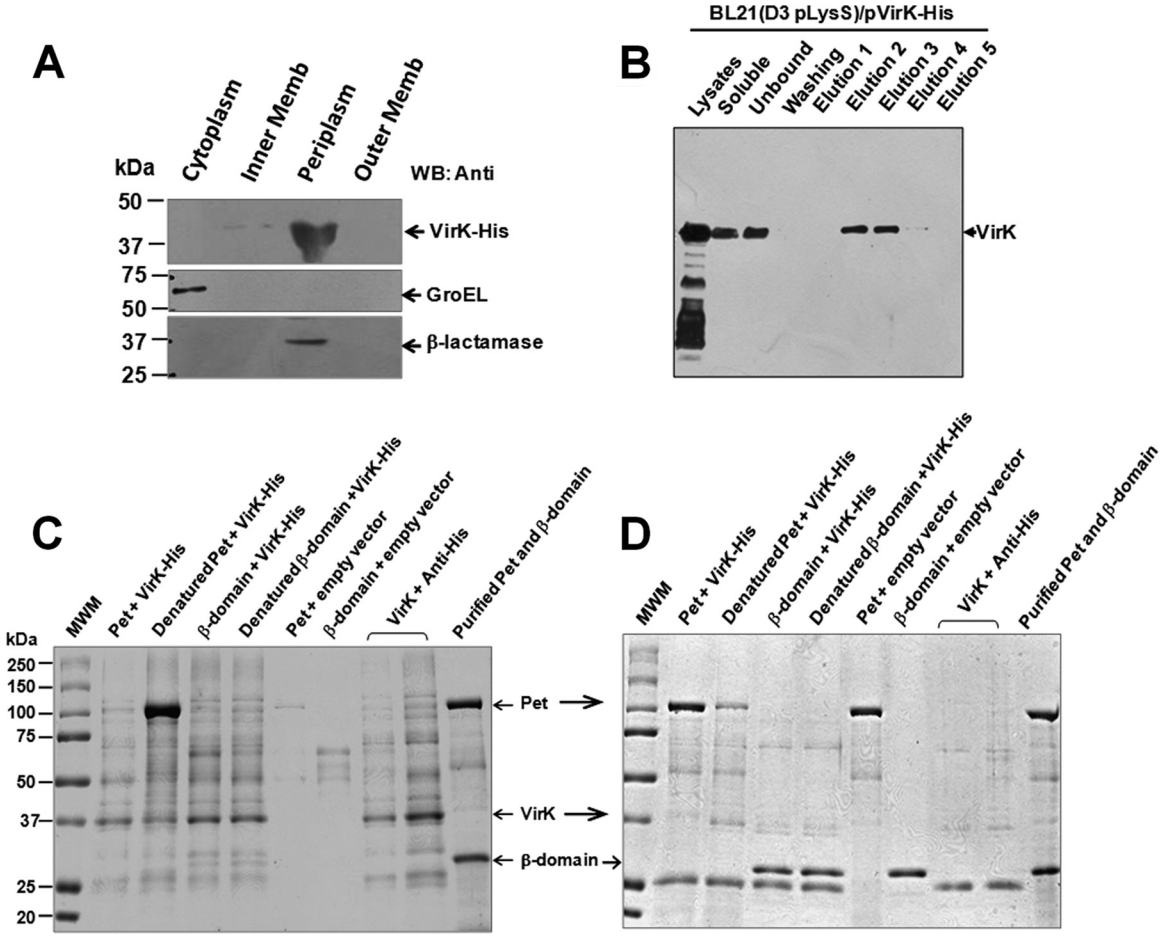

FIG 5 Affinity of Pet for purified VirK. (A) *E. coli* BL21 was fractionated as described in Materials and Methods. Cytoplasmic, IM, periplasmic, and OM fractions were analyzed by Western blot (WB) assay using anti-His antibodies and, as markers, antibodies against GroEL and β-lactamase. (B) Bacterial cultures transformed with pVirK-His were lysed 4 h after IPTG induction. VirK-His_6 was purified from cell lysates supernatant by Ni-NTA agarose affinity chromatography in accordance with the manufacturer's instructions (Qiagen). Fractions from different stages of the purification process were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. VirK-His_6 was visualized using a mouse anti-His primary antibody and an HRP-conjugated anti-mouse IgG secondary antibody. The arrow indicates the recombinant VirK protein. (C and D) Purified Pet, purified and denatured Pet, the purified β-domain, or the purified and denatured β-domain was incubated with VirK-His. Parallel samples were incubated with fractions 2 and 3 from BL21 transformed with the empty vector (pRSETA) as a negative control. After an overnight incubation, the complexes were immunoprecipitated with anti-His antibodies. As an additional control, the antibodies were incubated in the absence of either Pet or the β domain. The immunocomplexes (C) and nonimmunoprecipitated proteins (D) were separated by SDS-PAGE and visualized with Coomassie brilliant blue stain. MWM, MW markers.

tants (37) further indicates that VirK does not act as a general chaperone for OMPs.

To determine the fate of Pet which is not secreted in the absence of VirK, Western blot assays with an anti-Pet antibody were used to analyze the periplasmic fraction of EAEC and its isogenic Δ*virK* mutant (Fig. 6C). Pet was retained in the periplasm of the Δ*virK* mutant and degraded into peptides of different sizes. In contrast, the isogenic wild-type strain did not retain and degrade Pet to a substantial extent. These data indicated that VirK prevents the periplasmic degradation of Pet AT protein during secretion. The high-molecular-mass species (between 100 and 150 kDa) of Pet detected in EAECΔ*virK* represented unprocessed (i.e., passenger domain plus translocation domain) proteolytic products of the passenger domain, since antibodies against the β domain lightly recognized these species (data not shown).

VirK is required to produce the cytotoxic effect of EAEC. To determine if the absence of VirK blocks the secretion of Pet and thereby the cytotoxic effect of EAEC (1), we performed cytotoxicity assays of HEp-2 cells using wild-type EAEC, the EAECΔ*virK* mutant, and the *virK*-complemented EAECΔ*virK*/pVirK strain (Fig. 7). As assessed by confocal microscopy, HEp-2 cells infected with EAECΔ*virK* showed no damage to the actin cytoskeleton and were similar in appearance to mock-infected cells. In contrast, loss of actin stress fibers and cell rounding were observed in cells infected with wild-type EAEC or the complemented EAECΔ*virK*/pVirK strain. These results indicated that VirK is required for Pet secretion, which in turn induces the cytotoxic effect of EAEC on HEp-2 cells.

DISCUSSION

The name AT was coined to embody the idea that a single protein contains all of the functional elements necessary for its own secretion. However, recent work has suggested that many accessory proteins are actually active in AT secretion. Here we have shown

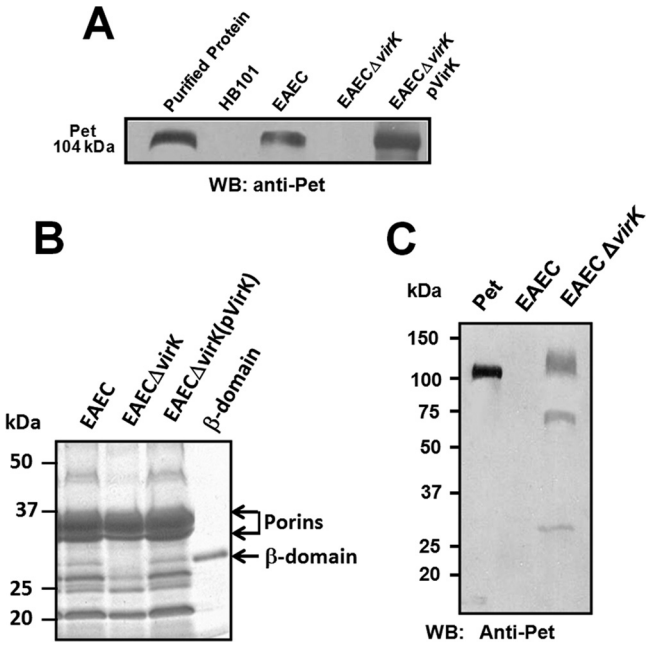

FIG 6 Inhibition of Pet AT but not porin secretion from *virK* null mutants. (A) Supernatants were collected from overnight cultures of an *E. coli* laboratory strain that does not express Pet (HB101), a pathogenic *E. coli* strain that expresses Pet (EAEC), isogenic strain EAECΔ*virK*, and *virK*-complemented strain EAECΔ*virK*/pVirK. Proteins in the supernatants were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The secreted pools of Pet were detected by Western blot (WB) analysis using a rabbit anti-Pet primary antibody, followed by an HRP-conjugated anti-rabbit IgG secondary antibody. (B) OMPs were obtained from cultures of EAEC, EAECΔ*virK*, and EAECΔ*virK*/pVirK; the purified β domain was used as a control. The OMPs were separated by SDS-PAGE and visualized with Coomassie brilliant blue stain. Arrows indicate the porins and the β-barrel translocation unit. For all experiments, supernatants from the bacterial strains were normalized by growth under equal conditions and sample harvesting at the same growth phase (optical density at 600 nm of 1.0) before precipitation with 20% TCA. (C) Retention and degradation of Pet AT protein in a *virK* mutant strain. Periplasmic fractions were obtained from overnight cultures of EAEC and its isogenic *virK* mutant. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The periplasmic pool of Pet was detected by Western blot analysis using a rabbit anti-Pet primary antibody, followed by an HRP-conjugated anti-rabbit IgG secondary antibody.

that the periplasmic protein VirK is needed to secrete Pet protein from EAEC. VirK was not required for the OM insertion of non-AT proteins. These results suggest that VirK is an accessory factor specifically involved with the biogenesis of a subset of secreted proteins such as the AT Pet.

Other accessory proteins are also known to operate in AT secretion. Release of the SPATE hemoglobin protease (Hbp) involves SurA and subunits of the BAM complex that promote the insertion and assembly of OM proteins (31). BamA (YaeT/Omp85), SurA, and Skp interact with the SPATE EspP (12). BamA (YaeT/Omp85) also appears to function in the OM assembly of multiple AT proteins (13). Finally, the DegP protease will degrade AT folding intermediates that accumulate in the periplasmic space (14). These observations support a model of AT biogenesis in which periplasmic proteins and the BAM complex collectively act to (i) prevent misfolding of the passenger domain before its secretion, (ii) catalyze the integration of the β domain into the OM, and (iii) facilitate the translocation of the passenger domain across the OM.

Here we report that the biogenesis of the AT Pet also involves the periplasmic protein VirK. Four periplasmic proteins with apparent molecular masses of 20, 37, 50, and 80 kDa were identified as Pet binding partners by coimmunoprecipitation, overlay, and pulldown studies. These proteins were identified with a strategy that involved delayed AT secretion as a means to capture the transient interactions between Pet and its chaperones. Similar strategies have been used to identify the roles of other periplasmic proteins in AT secretion (12, 14, 31). Protein sequencing identified the 20-kDa protein as Skp and the 37-kDa protein as VirK. Subsequent experiments using purified, His-tagged VirK confirmed the direct interaction between VirK and the Pet passenger domain. The 50- and 80-kDa Pet binding partners have yet to be identified, although it is interesting that SurA is a 48-kDa protein and BamA is an 86-kDa protein (12, 14, 31).

Skp bound to Pet in our coimmunoprecipitation assay, and an additional overlay assay indicated that Skp interacts specifically with the β-barrel translocation domain of Pet. This suggests that Skp, as previously reported for the AT Hbp (31), helps to insert the β-barrel domain into the OM. The collective data of the coimmunoprecipitation, overlay, and pulldown assays also indicate that Skp forms one component of a chaperone complex involving VirK, the 50-kDa protein, and the 80-kDa protein. However, a *skp* deletion mutant still secreted Pet into the medium and inserted the Pet β-barrel domain into the OM, albeit with lower efficiency than the isogenic wild-type strain. The release of Pet from MC4100Δ*skp* supports a model of AT biogenesis in which there are redundant, parallel pathways for AT secretion (28, 29).

The phenotype of the *virK* deletion strain in regard to bacterial growth and OMP insertion was distinct from the Δ*skp* mutant strain's phenotype and the previously described phenotypes of strains with other AT accessory factors deleted (27, 29). *surA* and *degP* mutant strains exhibited retarded growth rates and apparent cell lysis when expressing EspP (30). Furthermore, SurA, DegP, and Skp appear to function in the targeting and/or insertion of OMPs (28). In contrast, the Δ*virK* mutation did not affect growth rates (data not shown) and did not substantially inhibit the insertion of porin proteins into the OM. The lack of VirK interaction with the Pet β-barrel translocation domain also argues against a direct role for VirK in OM insertion. These collective observations suggest that the role of VirK in Pet AT biogenesis is distinct from the functions performed by AT accessory factors Skp, SurA, and DegP. The role(s) of VirK in the biogenesis of other classical (monomeric) ATs, as well as trimeric ATs and TpsA proteins, remains to be established.

Unlike Skp, VirK is essential for Pet secretion. The OM insertion of omptin IcsP (37) and porins was not disrupted in *virK* null mutants, but a *virK* deletion mutant of EAEC was unable to secrete Pet. The functional consequences of the Pet secretion defects were demonstrated with EAEC because EAECΔ*virK* did not display cytotoxicity against HEp-2 cells. Complementation of *virK* restored cytotoxicity and the mutant's capacity for secretion of the Pet passenger domain.

Multiple assays demonstrated that VirK recognizes misfolded/unfolded conformations of the Pet passenger domain but does not bind to the folded passenger domain, the native β domain, or the denatured β domain. However, OM insertion of the Pet β domain did not occur in the absence of VirK. This suggested that the folding state of the passenger domain during transit through the

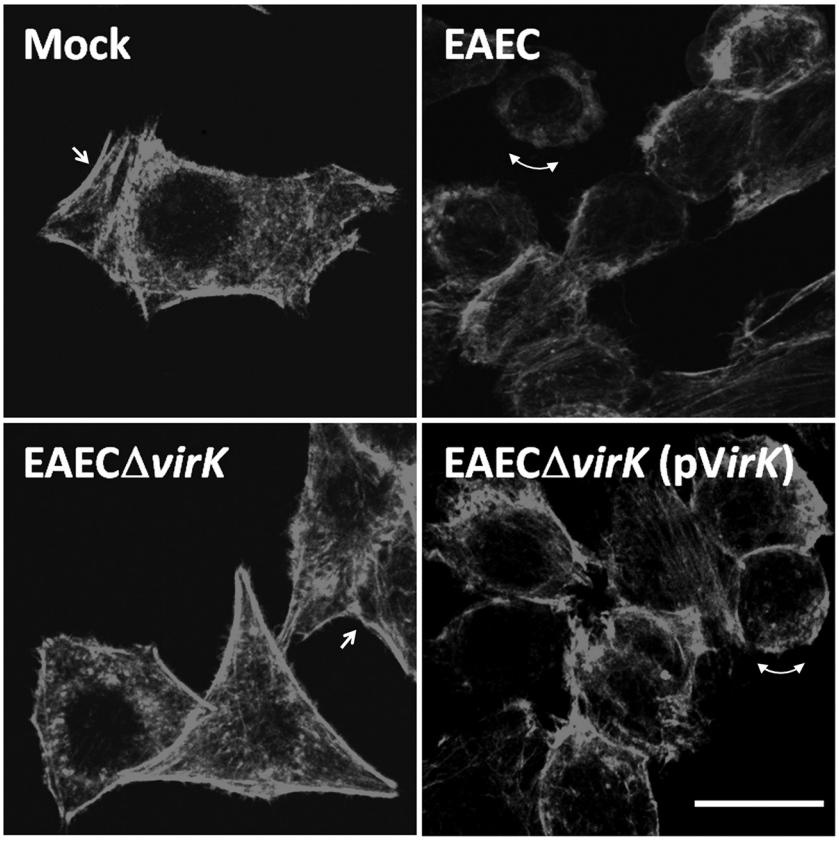

FIG 7 VirK is necessary for the cytotoxic effect of EAEC. HEp-2 cells were infected with EAEC, an isogenic EAECΔ*virK* mutant, and the *virK*-complemented strain EAECΔ*virK*/pVirK. Cells infected for 4 h were fixed, and the actin cytoskeleton was stained with rhodamine-phalloidin. Slides were observed by confocal microscopy. Uninfected cells (Mock) were also stained with rhodamine-phalloidin to establish the normal morphology of the actin cytoskeleton. Stress fibers are indicated with single-headed arrows; rounded cells are indicated with double-headed arrows. Bar, 20 μm.

periplasmic space can influence the efficiency of β-domain insertion into the OM. The detection of unprocessed Pet (i.e., passenger domain plus translocation domain) in the periplasm of EAECΔ*virK* further supports this conclusion. Thus, VirK interaction with the Pet passenger domain apparently assists the secretion process by both (i) directly preventing misfolding of the passenger domain before its secretion and (ii) indirectly assisting the integration of the β domain into the OM. Consistent with this model, proteolytic fragments of Pet accumulated in the periplasm of EAECΔ*virK*. The susceptibility of Pet to proteolysis during its biogenesis was also suggested by the higher levels of Pet recovered from the periplasm of protease-deficient *E. coli* UT5600 than from the periplasm of *E. coli* HB101. Collectively, our observations indicate a specific role for VirK in Pet secretion which involves maintaining the passenger domain in a conformation that avoids aggregation and proteolysis during transit through the periplasmic space.

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