THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 26, Issue of July 1, pp. 24663–24668, 2005
Printed in U.S.A.

Identification of an Essential Cleavage Site in ColE7 Required for Import and Killing of Cells*

Received for publication, February 2, 2005, and in revised form, April 27, 2005
Published, JBC Papers in Press, April 27, 2005, DOI 10.1074/jbc.M501216200

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Colicin E7 (ColE7), a nuclease toxin released from *Escherichia coli*, kills susceptible bacteria under environmental stress. Nuclease colicins are processed during translocation with only the cytotoxic nuclease domains traversing the inner membrane to cleave tRNA, rRNA, or DNA in the cytoplasm of target cells. In this study, we show that the *E. coli* periplasmic extract cleaves ColE7 between Lys^{446} and Arg^{447} in the presence or absence of its inhibitor Im7 protein. Several residues near cleavage sites were mutated, but only mutants of Arg^{447} completely lost *in vivo* cell-killing activity. Both the full-length and the nuclease domain of Arg^{447} mutants retained their nuclease activities, indicating that failure to kill cells was not a consequence of damage to the endonuclease activity of the enzyme. Moreover, the R447E ColE7 mutant was not cleaved at its 447 site by periplasmic extracts or transported into the cytoplasm of target cells. Collectively, these results suggest that ColE7 is cleaved at Arg^{447} during translocation and that cleavage is an essential step for ColE7 import into the cytoplasm of target cells and its cell-killing activity. Conserved basic residues aligned with Arg^{447} have also been found in other nuclease colicins, implying that the processing at this position may be common to other colicins during translocation.

Protein transport mechanisms across cell or organelle membranes have been studied extensively because these processes are essential for cell survival and defense. Protein toxins provide good opportunities for the study of protein import pathways into eukaryotic or prokaryotic cells because successful transport produces an obvious cell death consequence (1). The protein toxins specifically targeting bacterial cells are classified as bacteriocins. These toxins bind to the respective receptors on target cells and are transported across bacterial outer or, in some cases, inner cytoplasmic membranes, resulting in cell death (2). Presently, *Escherichia coli*-released colicins are likely the most studied subfamily of bacteriocins in terms of outer membrane receptor binding, membrane translocation, and cell killing (3–5).

Colicins are SOS response proteins, expressed under stress, that kill sensitive *E. coli* and other related bacterial strains (6). Most colicins share a similar organization containing three functional domains, the receptor-binding (R), membrane translocation (T), and cytotoxic (C) domains. After secretion from the host cell, colicins first bind to specific cell surface receptors on target cells. Examples include the vitamin B_{12} receptor BtuB for all the E-group colicins and iron siderophore receptor FepA for colicin B (ColB)[1] and colicin D (ColD) (7). They are then imported into cells by two different routes, one depending on Ton proteins (ExbB, ExbD, and TonB) and the other depending on Tol proteins (TolA, -B, -Q, and -R) (8, 9). Colicins use a variety of strategies to induce cell death through their C-domains. For example, pore-forming colicins create voltage-gated channels in the cytoplasmic membrane (4), and nuclease colicins cleave tRNA or rRNA at specific sites to inhibit protein synthesis (10, 11) or degrade nucleic acids nonspecifically in target cells (12, 13).

The crystal structures of two very different colicins, the Ton-dependent pore-forming ColIa (14) and Tol-dependent rRNase ColE3 (11), have revealed similarly assembled elongated Y-shaped molecules, with R-domains forming a long coiled-coil stalk and with the two globular heads of the T- and C-domains composing the two arms. In the ColE3-Im3 structure complex, the immunity protein Im3 is bound to the C-domain to prevent access of ColE3 to the ribosome and inhibits the rRNase activity of ColE3 upon ColE3 expression in the host cell. The crystal structure of the ColE3 R-domain in complex with its BtuB receptor further demonstrates how ColE3 interacts with BtuB at its coiled-coil apex to initiate colicin conformational change and translocation (15). However, the crystal structures of the Tol-dependent pore-forming colicin N (16) and the Ton-dependent pore-forming ColB (17) revealed different two-domain architectures. The dumbbell-shaped ColB has its T- and R-domains intertwining into a single large globular structure, suggesting that different colicins may have different mechanisms of translocation even though they use similar transporters.

After import into the periplasm, the nuclease colicins, containing rRNase, tRNase, or nuclease activities (DNase/RNase), have to transport further across the inner membrane to reach the cytoplasm of target cells. It has been shown that nuclease colicin ColE7 is likely processed in the periplasm during translocation with only the C-terminal cytotoxic nuclease domain transported into the cytoplasm (18). The tRNase colicin, ColD, was also reported to be cleaved during translocation and a leader peptidase, LepB, was identified as required in the processing (19). However, in contrast to ColE7, which is processed in the presence of the immunity protein Im7, the immunity protein of ColD was found to prevent ColD processing. It was thus suggested that the immunity protein may not only inhibit

* This work was supported by an Academia Sinica research grant and National Science Council of the Republic of China Grant NSC92-2321-B001-012 (to H. S. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Col, colicin; Ni-NTA, nickel-nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.

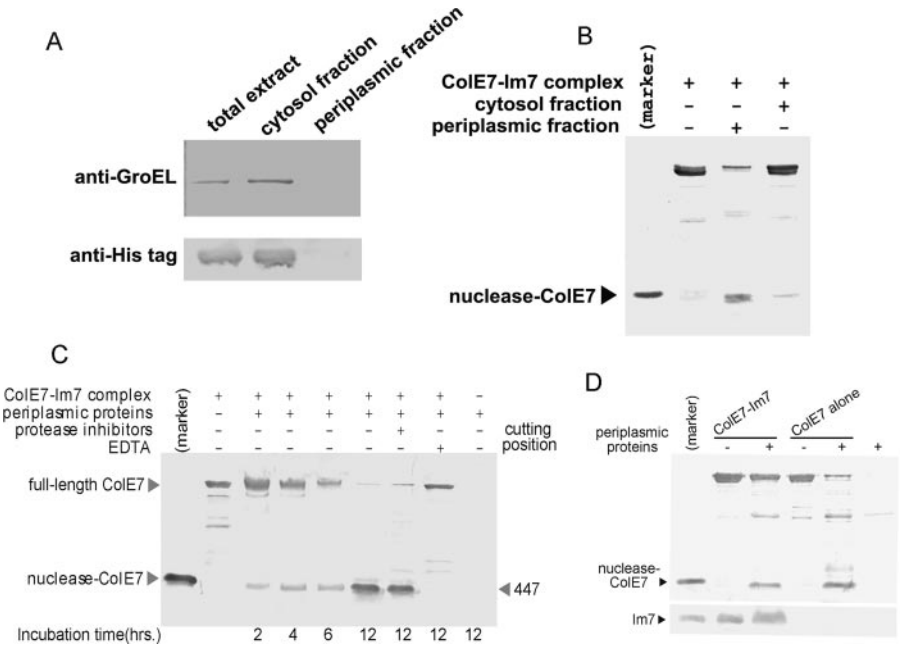

FIG. 1. **Processing of ColE7 by *E. coli* periplasmic extracts.** *A*, the periplasmic extract was prepared by osmotic shock using *E. coli* M15 cells containing a plasmid encoding His-tagged Im7. The quality of the periplasmic extract was confirmed by immunoblotting the cell fractions (3 μg each) with antibodies raised against the cytoplasmic markers GroEL (EMD Biosciences) and His-tagged Im7. GroEL and His-tagged Im7 were only identified in total cell and cytoplasmic fractions but not in periplasmic fractions. *B*, the ColE7-Im7 complex (3 μg) was incubated with cytoplasmic or periplasmic extracts at 37 °C for 12 h. ColE7 cleaved products were resolved by 12% SDS-PAGE followed by Western blot hybridization with antiserum raised against the C-domain of ColE7. The full-length ColE7 was mostly cleaved by the periplasmic fraction but was left almost intact by the cytoplasmic fraction. *C*, the ColE7-Im7 complex (3 μg) was further incubated with *E. coli* periplasmic extract (1 μg) for 2–12 h in the presence or absence of protease inhibitors and EDTA at 37 °C. A small fragment of ColE7 was generated by cleavage between Lys^{446} and Arg^{447} as determined by N-terminal sequencing. The addition of EDTA inhibited the processing, but the addition of serine or cysteine protease inhibitors did not inhibit the processing. *D*, the ColE7-Im7 complex and the free form of ColE7 were incubated with the periplasmic extract for 12 h. ColE7 was cleaved in the presence or absence of bound Im7.

tRNase activity but also protect ColD against LepB-mediated cleavage during export. Based on these results, it is generally accepted that nuclease colicins are processed during translocation but the cleavage sites in colicins and the component proteins involved in the processing have not yet been clearly elucidated. This report identifies the cleavage site at Arg^{447} in ColE7 during translocation and demonstrates that this cleavage is essential for ColE7 translocation and its cell-killing properties. A conserved basic residue was identified in a number of nuclease colicins, implying that a similar cleavage process may be involved for all these colicins during translocation.

MATERIALS AND METHODS

Protein Expression and Purification—DNA fragments encoding the full-length ColE7 and Im7 (20) and the C-domain of ColE7 and Im7 were first amplified by PCR and then subcloned between the SphI and BglII sites of pQE70. All mutants of ColE7-Im7 and C-domain-Im7 were generated using the QuikChange site-directed mutagenesis kits (Invitrogen). The *E. coli* strain M15 was used as the host strain for protein expression.

Full-length ColE7—Overnight cultures of *E. coli* cells were diluted 100-fold in 1 liter of LB containing 50 μg/ml ampicillin. Cells were grown at 37 °C to 0.6 O.D. (A_{600}), after which isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce protein expression. Crude cell extracts were first loaded onto a Ni-NTA resin affinity column (Qiagen) followed by a CarboxyMethyl column (Amersham Biosciences). Purified full-length ColE7-Im7 complexes were denatured with 6 M guanidine-HCl and further separated on a Ni-NTA spin column (Qiagen). The flow-through containing full-length ColE7 alone was dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.0) and 10 mM $ZnCl_2$.

ColE7 C-domain—ColE7 C-domain and Im7 fused with a His_6 affinity tag at the C terminus were expressed in the same *E. Coli* M15 strain. After Ni-NTA affinity column purification, the protein complex was denatured by dialysis against 20 mM glycine-HCl buffer (pH 3.0) overnight. The resulting protein solution was loaded onto a Sepharose-SP column (HiTrap SP, Amersham Biosciences) equilibrated with 20 mM glycine-HCl buffer (pH 3.0). The C-domain alone was eluted by an NaCl gradient (0–2.0 M, pH 3.0), and Im7 was eluted afterward by 20 mM sodium phosphate buffer (pH 7.0). The eluent containing the ColE7 C-domain was dialyzed against 20 mM sodium phosphate buffer (pH 7.0).

Preparation of Periplasmic Extracts—A published method was used for the preparation of periplasmic proteins by osmotic shock (21) with some modifications. Ten milliliters of fresh overnight *E. coli* (M15 strain) cell culture was diluted into 200 ml of LB and incubated at 37 °C for 5 h. Cells were harvested and suspended in 200 mM Tris-HCl buffer (pH 8.0) followed by an addition of the same volume of the buffer containing 200 ml Tris-HCl (pH 8.0) and 1 M sucrose. A final concentration of 0.5 mM EDTA was then added, resulting in a suspension containing ~5–20 mg/ml cells in 0.5 M sucrose. Lysozyme was then added into the cell suspension to a final concentration of 60 μg/ml. The same volume of water was then added to the cell suspension and the reaction kept for 10 min at room temperature. $MgSO_4$ was then added to a final concentration of 20 mM. The osmotic shock fluid containing periplasmic proteins was collected by centrifugation. The periplasmic extracts were further concentrated to 1 mg/ml by Centriprep (Millipore) followed by extensive dialysis against buffers containing 20 mM MES (pH 6.0) and 150 mM NaCl.

In Vitro Processing of ColE7—The purified ColE7-Im7 complex (3 μg) was mixed with 1 μg of the concentrated periplasmic extracts at pH 6.0 and incubated at 37 °C for various periods of time (~2–12 h). The processed protein products were fractionated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane using a semi-dry apparatus (American Bionetics). The membrane was hybridized with polyclonal antibodies raised against the ColE7 C-domain followed by goat anti-IgG antibodies conjugated with alkaline phosphatase (Promega). The blot was visualized by nitro blue tetrazolinm/5-bromo-4-chloro-3-indolyl phosphate (PerkinElmer Life Sciences). The desired fragment was eluted from a polyvinylidene difluoride membrane after staining with Coomassie Brilliant Blue and verified by N-terminal sequencing (492 Protein Sequencer, ABI).

In Vivo Cell Death Assay—Purified full-length wild-type or mutated ColE7-Im7 complexes were serially diluted in sodium phosphate buffers. A drop of 20 μl of ColE7-Im7 solution was spotted at each dilution onto a disk filter paper placed in freshly prepared bacterial lawns on

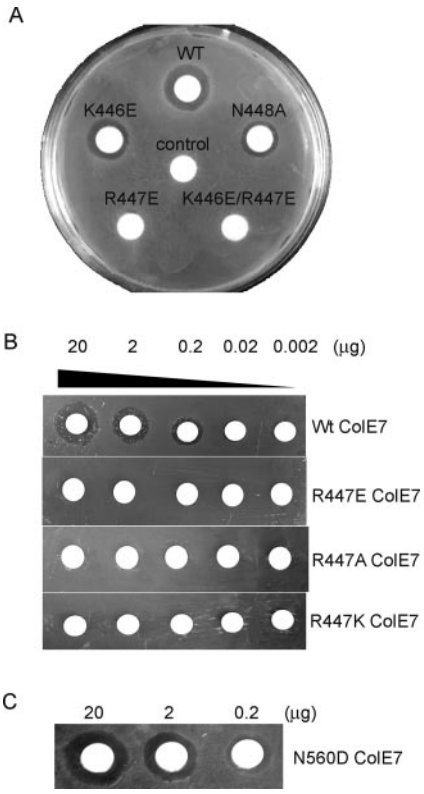

FIG. 2. **Arg^{447} mutants fail to kill sensitive *E. coli*.** *A*, a drop of 20 μl of wild-type or mutated ColE7-Im7 (2 mg/ml) was spotted onto a disk filter paper in an LB agar plate seeded with the *E. coli* M15 strain. Plates were then incubated overnight at 37 °C. Wild-type ColE7 killed bacteria and generated a clear circle surrounding the spotted site. N448A and K446E mutants gave reduced clear zones as compared with the results given by the wild-type ColE7. R447E and K446E/R447E failed to kill cells and gave no clear zone. *B*, wild-type (*Wt*) and Arg^{447}-mutated ColE7-Im7 were spotted onto agar plates in different amounts from 0.02 to 20 μg. All of the Arg^{447} mutants, R447E, R447A, and R447K, failed to kill ColE7-sensitive *E. coli*. *C*, the N560D ColE7-Im7 mutant was spotted onto the agar plates, and it induced cell death from 0.2 to 20 μg.

ampicillin LB agar plates. Plates were then incubated overnight at 37 °C. *E. coli* M15 containing pQE70 was used for the cell death assay.

Circular Dichroism and Tryptophan Fluorescence Spectroscopy—Circular dichroism measurements were performed at 25 °C using a Jasco J720 spectropolarimeter. The spectra presented in Fig. 3 were the average of three scans in units of mean residue ellipticity (θ). The protein concentration was 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0).

Measurements of the intrinsic tryptophan fluorescence emission of full-length ColE7-Im7 complex were performed on a Hitachi F4500 fluorescence spectrometer using an excitation wavelength of 295 nm at 25 °C. The excitation bandwidth was set at 5 nm and the emission bandwidth at 10 nm. The protein concentrations were 0.04 mg/ml each in 20 mM sodium phosphate (pH 7.0).

DNase Activity Assay—The purified full-length ColE7 (15 ng) was incubated with pUC18 plasmid in a final volume of 10 μl at 37 °C for ~2–3 h. The amount of ColE7 C-domain was first adjusted to between ~0.12 and 120 ng by series dilution and then incubated with the pQE70 plasmid at room temperature for 5 min. The plasmid digestion patterns were analyzed on 1% agarose gels.

DNase Activity Measured by Fluorescence Resonance Energy Transfer Methods—Thedouble-stranded DNA substrate labeled with fluorogenic material FAM (6-carboxyl-fluorescein) and TAMRA (6-carboxyl-tetramethyl-rhodamine) at the 5′ end was prepared by Biotech (Taiwan).

FAM-5′-CCACAGGTAGCGACAG-3′

3′-GGTGTCCATCGCTGTC-5′-TAMRA

SEQUENCE I

Kinetic measurements were performed in 10 mM Tris (pH 8.0) and 1 mM $MgCl_2$ at 25 °C. Enhanced fluorescence, a result of cleavage of the fluorogenic double-stranded DNA substrate, was monitored at 515 nm, with excitation at 486 nm using a fluorescence plate reader (PerkinElmer 1420 Victor2 mutilabel counter). The enzyme concentration used in measuring K_m and k_{cat} values was 20 nM with varied substrate concentrations of 9.75, 19.14, 47.85, 71.78, 95.7, 143.55, and 191.4 nM.

Identification of ColE7 Translocation Products—A method reported previously (18) was used to extract the final processed ColE7 from the cytoplasm of target cells. A cell culture of 200 ml of *E. coli* M15 containing a plasmid encoding His-tagged Im7 (pQE30-*cei*) was treated with 5 mg of ColE7-Im7 complex for 4 h. The treated cell culture was harvested and washed twice with 20 mM sodium phosphate buffer (pH 7.0) followed with 10 mg/ml proteinase K for 1 h at 37 °C to remove residual proteins bound on the cell surface. The proteinase K-treated cells were harvested and washed twice with the same buffer and then resuspended in 3 ml of sodium phosphate buffer (50 mM, pH 7.0) containing 300 mM NaCl and protease inhibitor complex (Roche Complete, EDTA-free). Cells were disrupted by sonication and the insoluble fractions removed by centrifugation. The soluble fractions were then loaded onto a Ni-NTA spin column (Qiagen). The His-tagged Im7 and any pull-down proteins from the spin column were eluted by 500 mM imidazole. Im7-associated proteins were identified by Western blot hybridization with polyclonal antibodies against the C-domain of ColE7.

RESULTS

Periplasmic Proteins Cleave ColE7 at Arg^{447} in the Presence or Absence of Im7—To ascertain the location of ColE7 processing sites, a full-length ColE7-Im7 complex was incubated with *E. coli* periplasmic extracts prepared by osmotic shock (21). The quality of the periplasmic extract prepared from the *E. coli* M15 strain containing a plasmid encoding His-tagged Im7 was checked by Western blot hybridization using the cytoplasmic antibody markers against GroEL and His tags. Both GroEL and His-tagged Im7 were detected in total cell extracts and cytoplasmic fractions but were not detected in periplasmic fractions, indicating that the prepared periplasmic fraction was not contaminated by cytoplasmic proteins (Fig. 1*A*). After incubation with the cell fractions for 12 h, ColE7 was mostly cleaved by the periplasmic extract but not by cytoplasmic fractions (Fig. 1*B*). The ColE7-Im7 was then further incubated with periplasmic extracts over different periods of time (2–12 h) in the presence or absence of metalloprotease inhibitors and EDTA. The processed ColE7 products were separated using SDS-PAGE and detected by Western blot hybridization using an antibody raised against the ColE7 C-domain, revealing a processed fragment with a size similar to the constructed nuclease domain (Fig. 1*C*). The addition of EDTA but not serine or cysteine protease inhibitors inhibited the processing, implying that the processing might involve metalloproteases.

The processed ColE7 product was then extracted from the SDS-PAGE and analyzed by N-terminal sequencing, revealing the first five residues of the processed ColE7 as RNKPG, corresponding to residues Arg^{447} to Gly^{451}. Therefore the ColE7 cleavage site lay between Lys^{446} and Arg^{447}, located in a region between its R-domain and C-domain. By incubating the free form of ColE7 (without Im7) with periplasmic extracts, we found the processing pattern (Fig. 1*D*) similar to that of the ColE7-Im7 complex. This result shows that periplasmic extracts cleave ColE7 between Lys^{446} and Arg^{447} in the presence or absence of bound Im7.

Arg^{447} ColE7 Mutants Lose in Vivo Cell-killing Activity—Several single- or double-point ColE7 mutants were constructed to determine which of the amino acids flanking the Lys^{446}-Arg^{447} cleavage site, including K446E, R447E, N448A, and K446E/R447E, would affect cleavage. In the cell death assay, wild-type ColE7-Im7, spotted on a disk filter paper placed in an agar plate seeded with *E. coli* M15 strains, killed cells and gave a clear region around the filter paper. However, two of the mutants with mutations at the 447 site, R447E and

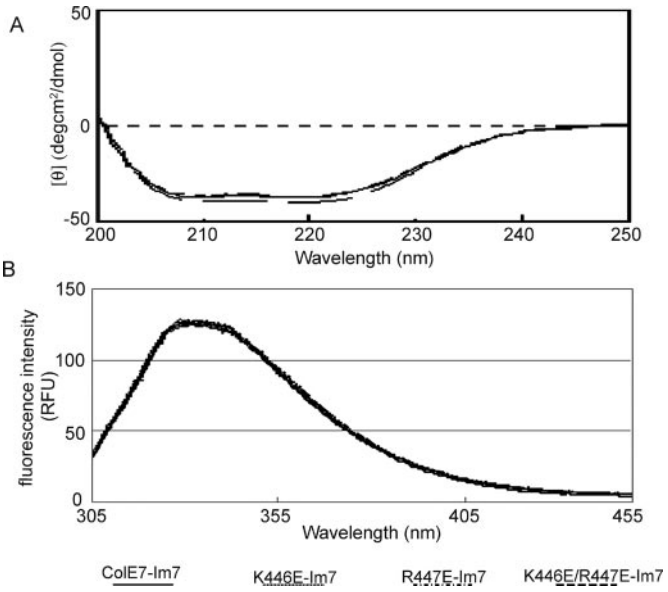

FIG. 3. **Circular dichroism and tryptophan fluorescence spectra of wild-type and Arg^{447}-mutated ColE7-Im7.** *A*, the circular dichroism spectra of the wild-type ColE7-Im7 (——), K446E-Im7 (·· ··), R447E-Im7 (–·–), and K446E/R447E-Im7 (– – –) were recorded from 200 to 250 nm with protein concentrations of 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0) at 25 °C. *B*, the fluorescence intensity of wild-type and mutated ColE7-Im7 shown in relative fluoresence units (*RFU*) were measured using an excitation wavelength of 295 nm at 25 °C. Protein concentrations were 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0).

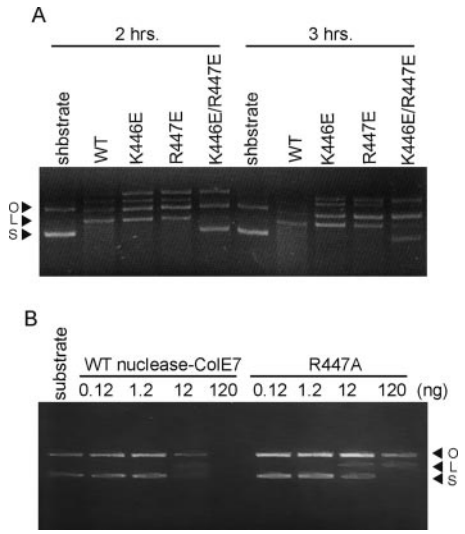

FIG. 4. ***In vitro* endonuclease activity assay for wild-type (*WT*) and mutated ColE7.** *A*, plasmid pUC18 (300 ng) was incubated with 15 ng of full-length ColE7 proteins for 2 or 3 h at 37 °C. The digested DNA was resolved by agarose gel (1%) electrophoresis. Supercoiled (*S*) plasmid DNA was cleaved into linear (*L*) or open-circular (*O*) forms by the wild-type and ColE7 mutants (K446E, R447E, and K446E/R447E). *B*, various amounts (0.12–120 ng) of wild-type ColE7 C-domain (residues 444–576) and the corresponding R447A mutant were incubated with 300 ng of plasmid pQE70 for 5 min at room temperature. The R447A C-domain had ~10-fold lower endonuclease activity compared with the wild-type C-domain.

K446E/R447E, failed to kill target bacteria, as evidenced by the lack of a clear region surrounding the disk (Fig. 2*A*). In contrast, K446E and N448A mutants retained significant cell-killing activities. Arg^{447} was then further mutated to a similar basic residue (Lys) or to a noncharged residue (Ala). However, neither R447K nor R447A killed any cells even upon application at high concentrations (Fig. 2*B*). This result demonstrates the critical importance of Arg^{447} in mediating the cell-killing

TABLE I
The endonuclease activity of the wild-type and mutated nuclease domain of ColE7 as measured by the fluorescence resonance energy transfer method

Protein	k_{cat}	K_m	k_{cat}/K_m	Relative activity toward a 16-bp dsDNA[a]
	$10^{-3}/s^{-1}$	nM	$10^4\ M^{-1}\ s^{-1}$	%
Wild type[a]	1.81	27.25	1.95	100
R447A	1.09	105.50	0.30	15.4
N560A	0.02	144.00	0.014	0.7

[a] Double-stranded DNA.
[b] The wild-type nuclease domain of ColE7 contains residues from 444 to 576.

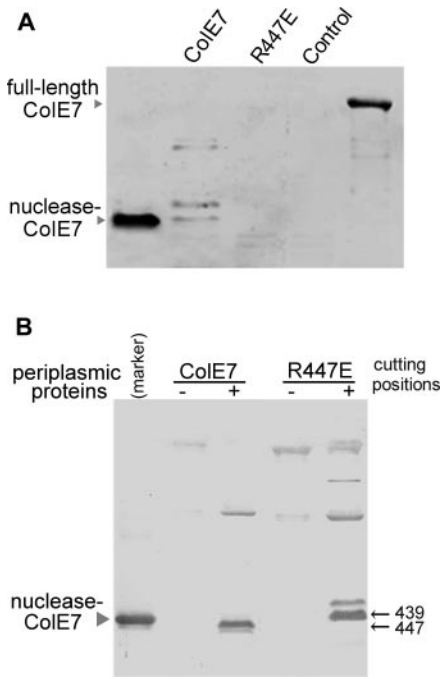

FIG. 5. **R447E ColE7 mutant is not imported into the cytoplasm of *E. coli* cells and is cleaved at different sites by a periplasmic extract.** *A*, a cell culture of 200 ml of *E. coli* M15 containing a plasmid encoding His-tagged Im7 (pQE30-*cei*) was treated with wild-type ColE7-Im7 (5 mg) and then with Arg^{447}-Im7 (5 mg) for 4 h. The proteinase K-treated cells were then harvested, and the processed ColE7 bound to cytoplasmic His-tagged Im7 was purified by Ni-NTA chromatography. Western blots with polyclonal antibodies against the C-domain of ColE7 identified processed ColE7 fragments in cells treated with wild-type ColE7-Im7. However, no ColE7 fragments were identified in cells treated with R447E-Im7. Full-length and C-domain ColE7 proteins were used as markers. A control of M15 cells was treated with only sodium phosphate buffer. *B*, ColE7-Im7 and R447E-Im7 were treated with the periplasmic extract, and the ColE7-processed fragments were resolved by 12% SDS-PAGE followed by Western blot. The ColE7 R447E mutant was not processed at the 447 position, but a larger fragment with a cleavage between Lys^{438} and Ala^{439} was observed.

activity of ColE7 *in vivo*. Circular dichroism and tryptophan fluorescence spectroscopy were used to detect any conformational changes in Arg^{447} mutants (Fig. 3). No obvious differences were detected in secondary and tertiary structures between wild-type ColE7 and Arg^{447} mutants, indicating that the failure to kill cells, as exhibited by Arg^{447} mutants, was likely not to be because of any change in protein conformation.

Arg^{447} ColE7 Mutants Retain DNase Activity—Because it was possible that the Arg^{447} mutants lacked cell lethality as a result of losing their endonuclease activity, full-length wild-type and mutated ColE7 proteins were prepared and purified for nuclease activity assays. Plasmids pUC18 or pQE70 were used as substrates to monitor ColE7 endonuclease activity from any DNA topological changes seen in agarose gel electrophoresis. Fig. 4*A* shows that the supercoiled plasmid was cleaved

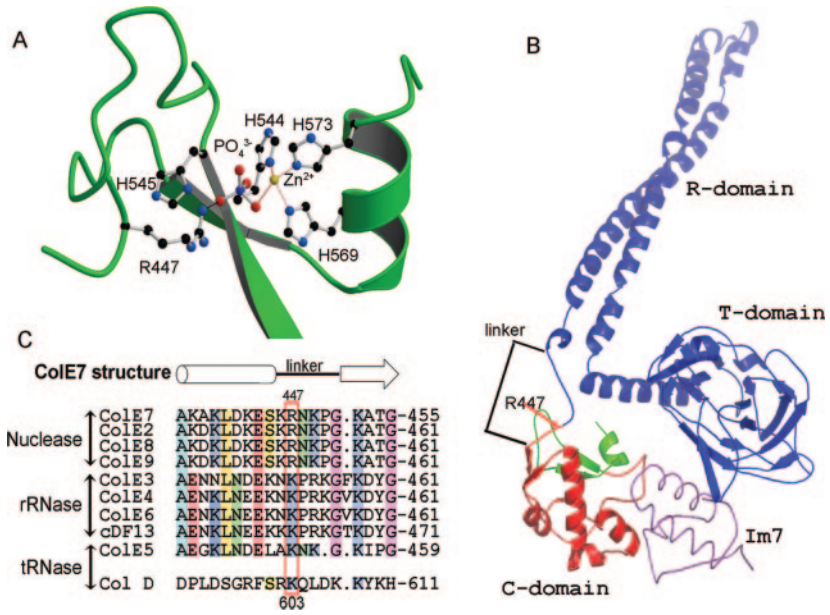

FIG. 6. **A structural model of ColE7 and sequence alignments of nuclease colicins.** *A*, the structure of the HNH motif in the C-domain of ColE7 (Protein Data Bank accession number 1MZ8) demonstrates that Arg^{447} binds directly to the phosphate. Only the phosphate ion and the side chains of Arg^{447}, His^{545}, His^{544}, His^{569}, and His^{573} are displayed in the *ball-and-stick model*. *B*, structural model of full-length ColE7-Im7 is shown in a *ribbon model*. The structure was constructed by fusing ColE3 T- and R-domains (Protein Data Bank accession number 1JCH, in *blue*) with a ColE7 C-domain-Im7 (Protein Data Bank accession number 7CEI). The C-domain of ColE7 is displayed in *red* with only the HNH motif in *green*. The ColE7 inhibitor Im7 is displayed as a *magenta coil structure*. Arg^{447} is located at the linker region between R- and C-domains. *C*, sequence alignments of several nuclease colicins near their linker regions. All of the Tol-dependent nuclease colicins share high sequence homology with ColE7 at the linker region and have a basic residue, arginine or lysine, that aligns with Arg^{447} in ColE7.

into linear/open circular forms by either wild-type or mutated ColE7 (K446E, R447E, and K446E/R447E). The wild-type nuclease domain of ColE7 (residues 444–576) and the corresponding R447A mutant were further constructed and purified. These two C-domain proteins also cleaved the plasmid substrate efficiently (Fig. 4*B*). Nevertheless, the R447A C-domain had ~10-fold lower endonuclease activity than the wild-type protein.

The catalytic activities of wild-type and mutant C-domains were further measured by a fluorescent method using a fluorophore and quencher-labeled oligonucleotide as the substrate. For a control to compare with Arg^{447} mutants, a mutant, N560D, was selected for the measurement of enzyme and cell-killing activity. Asn^{560} is a conserved residue in the *HNH* motif located in the C-domain of ColE7; therefore, the mutant N560D was expected to have reduced endonuclease activity. Cleavage of the fluorophore-labeled oligonucleotides by the C-domain proteins gave increasing fluorescence emission intensities. The measured intensities showed that R447A C-domain had 15% and N560D C-domain had 0.7% of wild-type enzyme activity (Table I). The reduced catalytic activity of R447A was not because of any decrease in enzyme activity but mostly resulted from an increased K_m, indicating that the affinity between R447A and DNA was lower than that of the wild-type enzyme. N560D mutant, with a much lower overall endonuclease activity of only 0.7%, was still able to kill target cells (Fig. 2*C*), indicating that any failure to kill cells by Arg^{447} mutants did not result from any reduction in endonuclease activity.

ColE7 R447E Mutants Are Not Imported into Cytoplasm or Processed at the 447 Site—To further elucidate the underlying cause for the failure of Arg^{447} mutants to kill cells, the imported ColE7 in the cytoplasm of target cells was characterized. *E. coli* cells containing an endogenously expressed His-tagged Im7 were treated with full-length wild-type ColE7-Im7 and R447E-Im7 complexes. The translocation product in complex with the endogenous His-tagged Im7 was then purified by Ni-NTA resin and detected by Western blot hybridization using an antibody against nuclease-ColE7. Fragments of nuclease domains were detected in target cells treated with wild-type ColE7-Im7 complex. However, no nuclease domain fragments were detected in the cells treated with R447E-Im7 complex (Fig. 5*A*). This result indicates that the ColE7 R447E mutant was not imported into the cytoplasm of target cells in the same manner as that of wild-type ColE7.

It was intriguing that ColE7 Arg^{447} mutant was not imported into the cytoplasm of target cells. It is thus necessary to determine whether R447E could be processed correctly in periplasm, because the incorrect processing may lead to the failure in import. Full-length ColE7 R447E mutant was incubated with periplasmic extracts, and the digested protein products were separated in SDS-PAGE and detected by Western blot hybridization (Fig. 5*B*). The R447E mutant was not cleaved between Lys^{446} and Glu^{447}. However, a larger fragment was found, resulting from a cut between Lys^{438} and Ala^{439} as analyzed by the N-terminal sequencing. This result suggests that cleavage at Arg^{447} is necessary for ColE7 import into target cells, and that is why the mutant R447E, not processed at 447, was not imported into the cytoplasm.

DISCUSSION

Specific Cleavage at Arg^{447} in ColE7 Is Required for Cell-killing Activity—The nuclease colicins acting in the cytoplasm of sensitive bacterial cells have to traverse the second inner membrane, and as a result they require additional steps in translocation as compared with pore-forming colicins. Here we present evidence demonstrating that cleavage between Lys^{446} and Arg^{447} in ColE7 is essential for its import into the cytoplasm of sensitive cells. The arginine at the P1′ site (cleavage between P1 and P1′) is of critical importance, because replacing it with other residues abolished the specific cleavage and protein translocation, resulting in a non-lethal colicin. This cleavage is independent of immunity protein, *i.e.* Im7 cannot protect ColE7 from this cleavage. This cleavage process occurs extracellularly, most likely in the periplasm of sensitive cells. How-

ever, because outer membrane-associated proteins may have also been extracted under the preparation conditions used in the isolation of periplasmic extracts, it cannot be excluded that cleavage may take place during ColE7 translocation across the outer membrane.

If one compares Arg^{447} cleavage in ColE7 with the LepB-mediated cleavage in ColD during translocation (19), similar features can be found. Firstly, the cleavages of ColD and ColE7 are required for their import into cytoplasm and for their cell-killing activity. Secondly, several single, double, and triple mutants have been constructed for ColD, but only the mutants in which the basic residue Lys^{603} was replaced (R602L/K603E and R602L/K603E/L596P) were not cleaved, similar to the Arg^{447} mutants in ColE7. However, cleavage of ColD by whole cell extracts was inhibited by the immunity protein and mediated by an inner-membrane-associated peptidase (19), which was not present in the periplasmic extract used in this analysis. Moreover, the signal peptidase LepB is a serine protease but not a metalloprotease. However, the processing of ColE7 by periplasmic extracts is inhibited by EDTA, indicating that a metalloprotease may be involved. Therefore, it is likely that the observed cleavages in ColE7 and ColD occur at different stages or are processed by different proteins during translocation. The difference observed in processing may also be a consequence of two different types of colicins using two different translocation pathways for import: the nuclease ColE7 depending on Tol proteins and tRNase ColD depending on TonB.

Cleavage Site in ColE7 Is Located in a Linker Region between R-domain and C-domain—The crystal structure of an E-group rRNase colicin, ColE3, has been determined. ColE3 shares high sequence identity with ColE7 in the T- and R-domains (75.4% identity). We therefore constructed a ColE7 structure model by fusing the crystal structure of ColE3 T- and R-domain (Protein Data Bank accession number 1JCH) to the crystal structure of a ColE7 C-domain (the nuclease domain) in complex with Im7 (Protein Data Bank accession number 7CEI) (11, 22). The overall structure and a closer view of the endonuclease active site in ColE7 are shown in Fig. 6. In the crystal structure of ColE7 C-domain (Protein Data Bank accession number 1MZ8), Arg^{447} hydrogen bonds to the phosphate ion mimicking the scissile phosphate that is directly bound to the metal ion in the active site (23). This explains why the R447A mutant has a lower affinity for DNA substrates even though it retains endonuclease activity, because Arg^{447} indeed is involved in DNA binding but not directly involved in catalysis.

The location of Arg^{447} in the exposed linker region seems ideal for protease processing. This site is distant from Im7, which is consistent with the fact that Im7 cannot protect ColE7 from cleavage. From comparisons of ColE7 sequence with those of other nuclease colicins, the conserved basic residues arginine or lysine are seen to align at the same position as Arg^{447} for all the Tol-dependent colicins. The E-group rRNase (E3, E4, E6) and tRNase (E5) colicins, all have a lysine, whereas the non-specific nuclease colicins (E2, E7, E8, and E9) all have an arginine at this position (Fig. 6*C*, *red box*). The Tol-dependent DF13, which binds to the receptor IutA (24), also shares high sequence homology with the E-group colicins in this linker region, and DF13 also has a lysine aligned with Arg^{447} in ColE7. This implies that the cleavage observed in ColE7 at Arg^{447} may be common to other Tol-dependent enzymatic colicins. The Ton-dependent nuclease colicin ColD shares low sequence homology with ColE7. However, it will be interesting to learn whether ColD requires cleavage at Lys^{603} for translocation. Additional experiments are needed to find out whether the processing seen for ColE7 at the Arg^{447} site is general for the import of all enzymatic colicins and the protein components involved in this process.

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