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

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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 Lys446 and Arg447 in the presence or absence of its inhibitor Im7 protein. Several residues near cleavage sites were mutated, but only mutants of Arg447 completely lost in vivo cell-killing activity. Both the full-length and the nuclease domain of Arg447 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 Arg447 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 Arg447 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 B12 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 induce 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 rRNAse 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

1 The abbreviations used are: Col, colicin; Ni-NTA, nickel-nitrilotriacetic acid; MES, 4-morpholineethanesulfonic acid.
Specific Cleavage at ColE7 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. (A600), after which isopropyl 1-thio-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 complexes (5 μg) were incubated with cytoplasmic or periplasmic extracts for 5 h. Cells were harvested and suspended in 200 mM Tris-HCl buffer (pH 8.0). The eluent containing the ColE7 C-domain was dialyzed against 20 mM sodium phosphate buffer (pH 7.0). 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.

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 mM 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. MgSO4 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 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 100-fold in 1 liter of LB containing 50 μg/ml ampicillin. Cells were grown at 37 °C to 0.6 O.D. (A600), after which isopropyl 1-thio-D-galactopyranoside was added to a final concentration of 1 mM to induce protein expression. Crude cell extracts were then loaded onto a Ni-NTA resin affinity column (Qiagen) followed by an addition of the same volume of the buffer containing 20 mM sodium phosphate buffer (pH 7.0). 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.

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ampicillin LB agar plates. 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 mutants failed to kill sensitive E. coli cells and gave no clear zone. B, wild-type (Wt) and Arg447 mutants were spotted onto agar plates in different amounts from 0.02 to 20 μg. All of the Arg447 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.

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K446E/R447E, failed to kill target bacteria, as evidenced by the lack of a clear region surrounding the disk (Fig. 2A). In contrast, K446E and N448A mutants retained significant cell-killing activities. Arg447 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. 2B). This result demonstrates the critical importance of Arg447 in mediating the cell-killing activity of ColE7 in vivo. Circular dichroism and tryptophan fluorescence spectroscopy were used to detect any conformational changes in Arg447 mutants (Fig. 3). No obvious differences were detected in secondary and tertiary structures between wild-type ColE7 and Arg447 mutants, indicating that the failure to kill cells, as exhibited by Arg447 mutants, was likely not to be because of any change in protein conformation.

**Arg447 ColE7 Mutants Retain DNase Activity**—Because it was possible that the Arg447 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. 4A shows that the supercoiled plasmid was cleaved activity of ColE7 in vivo. Circular dichroism and tryptophan fluorescence spectroscopy were used to detect any conformational changes in Arg447 mutants (Fig. 3). No obvious differences were detected in secondary and tertiary structures between wild-type ColE7 and Arg447 mutants, indicating that the failure to kill cells, as exhibited by Arg447 mutants, was likely not to be because of any change in protein conformation.

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### Table I

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ (nM)</th>
<th>$k_{cat}/K_m$</th>
<th>Relative activity toward a 16-bp dsDNA $^{a}$</th>
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<tr>
<td>Wild type $^{b}$</td>
<td>1.81</td>
<td>27.25</td>
<td>1.95</td>
<td>100</td>
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<tr>
<td>R447A</td>
<td>1.09</td>
<td>105.50</td>
<td>0.30</td>
<td>15.4</td>
</tr>
<tr>
<td>N560A</td>
<td>0.02</td>
<td>144.00</td>
<td>0.014</td>
<td>0.7</td>
</tr>
</tbody>
</table>

$^{a}$ Double-stranded DNA.

$^{b}$ The wild-type nuclease domain of ColE7 contains residues from 444 to 576.
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. 4B). 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 fluorphore and quencher-labeled oligonucleotide as the substrate. For a control to compare with Arg447 mutants, a mutant, N560D, was selected for the measurement of enzyme and cell-killing activity. Asn560 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 fluorphore-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. 2C), indicating that any failure to kill cells by Arg447 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 Arg447 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. 5A). 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 Arg447 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. 5B). The R447E mutant was not cleaved between Lys446 and Glu447. However, a larger fragment was found, resulting from a cut between Lys438 and Ala439 as analyzed by the N-terminal sequencing. This result suggests that cleavage at Arg447 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 Arg447 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 Lys446 and Arg447 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 Arg447 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 Lys803 was replaced (R602L/K603E and R602L/K603E/L596P) were not cleaved, similar to the Arg447 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 nucleic 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), Arg447 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 Arg447 indeed is involved in DNA binding but not directly involved in catalysis.

The location of Arg447 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 Arg447 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. 6C, 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 Arg447 in ColE7. This implies that the cleavage observed in ColE7 at Arg447 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 Lys803 for translocation. Additional experiments are needed to find out whether the processing seen for ColE7 at the Arg447 site is general for the import of all enzymatic colicins and the protein components involved in this process.

REFERENCES