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High-resolution Crystal Structure of a Truncated ColE7 Translocation Domain: Implications for Colicin Transport Across Membranes

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ColE7 is a nuclease-type colicin released from *Escherichia coli* to kill sensitive bacterial cells by degrading the nucleic acid molecules in their cytoplasm. ColE7 is classified as one of the group A colicins, since the N-terminal translocation domain (T-domain) of the nuclease-type colicins interact with specific membrane-bound or periplasmic Tol proteins during protein import. Here, we show that if the N-terminal tail of ColE7 is deleted, ColE7 (residues 63–576) loses its bactericidal activity against *E. coli*. Moreover, TolB protein interacts directly with the T-domain of ColE7 (residues 1–316), but not with the N-terminal deleted T-domain (residues 60–316), as detected by co-immunoprecipitation experiments, confirming that the N-terminal tail is required for ColE7 interactions with TolB. The crystal structure of the N-terminal tail deleted ColE7 T-domain was determined by the multi-wavelength anomalous dispersion method at a resolution of 1.7 Å. The structure of the ColE7 T-domain superimposes well with the T-domain of ColE3 and TR-domain of ColB, a group A Tol-dependent colicin and a group B TonB-dependent colicin, respectively. The structural resemblance of group A and B colicins implies that the two groups of colicins may share a mechanistic connection during cellular import.

Keywords: protein cellular import; membrane translocation; colicin structure; colicin translocation domain; crystal structure

Introduction

Protein transport across membranes is a general event occurring in all kinds of cells, such as protein import into chloroplast and mitochondria in eukaryotic cells, and polypeptide import into or export from bacterial cells. With respect to protein import into bacteria, colicins have been studied extensively in outer membrane receptor binding and membrane translocation.[1–3] Colicins are protein toxins released from *Escherichia coli* to kill other closely related bacteria strains during environmental stress.[4] They usually share a similar organization, containing three functional domains, receptor-binding (R), translocation (T) and cytotoxic (C). Some colicins kill sensitive bacteria by forming ion channels to depolarize the inner membrane of bacteria.[5] These pore-forming colicins, containing a channel-forming C-domain, transport across only the outer membrane into the periplasm after binding to specific cell receptors with their R-domains. On the other hand, some enzymatic colicins bearing an RNase or a nuclease C-domain kill bacteria by either cleaving ribosomal or transfer RNA, or degrading chromosomal DNA in the cytoplasm.[6] This class of enzymatic colicins has to traverse both outer and inner membranes to import their C-domain into the cytoplasm of sensitive cells to reach their target substrates.

Based on the different proteins used during translocation across membranes, colicins are classified into groups A and B. Group A colicins use the

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Abbreviations used: MAD, multi-wavelength anomalous dispersion; rmsd, root-mean-squared difference; Se-Met, selenomethionine; PDB, Protein Data Bank.

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Tol/Pal system, which consists of five proteins: inner membrane-associated TolQ, TolR and TolA, periplasmic TolB and outer membrane anchored lipoprotein Pal.[7] TolB interacts with Pal and TolA, hence Tol/Pal proteins form a complex linking the inner and outer membranes, likely playing a role in the maintenance of cell envelope integrity.[8] It has been shown that the N-terminal tail in the T-domain of group A colicins, including colicins A,[9] E3[10] and E9,[11] directly interacts with TolB by random mutagenesis, *in vivo* competition assays, cross-linking experiments and yeast two-hybrid systems. A homologous "TolB box" with a sequence of DGSGW has been identified in colicin A (residues 11–18), and colicins E2 to E9 (residues 35–39), that is suggested to interact with TolB.[9,11] Moreover, it has been shown that TolA interacts with distinct regions of the N-terminal tail in colicins A, E1 and N;[9,12,13] TolR interacts with the very N-terminal end of colicin A.[14] Therefore, group A colicins appear to make multiple contacts with various periplasmic-exposed Tol proteins during translocation. Group B colcins use the Ton system, consisting of TonB, ExbB (homologous to TolQ) and ExbD (homologous to TolR), which are normally involved in the uptake of siderophores and vitamin B12.[15] Less is known about the protein import mechanism of group B colicins; however, a conserved TonB box sequence has been identified in group B colicins and it is suspected that this sequence in the N-terminal domain of colicins binds to TonB.[16]

Crystal structures of two group A colicins, E3[17] and N,[18] and two group B colicins, Ia[19] and B,[20] have been resolved in (or closely in) full length. Colicins Ia and E3, although belonging to different groups, share similar structural organization, each having three separate structural domains assembled in a Y shape: the long stalk-like coiled-coil R-domain and two globular arm-like T and C-domains. On the contrary, colicins N and B only contain two domains folded in a dumbbell shape, with a pore-forming C-domain located at one side of a long α-helix and an intertwined TR-domain located at the other side. It seems that these group A and B colicins share some structural similarity in overall architecture but the similarity does not correlate with the proteins they use for translocation across membranes.

This study used ColE7 as a model system to investigate the amino acids and structure required for ColE7 protein import into bacteria. ColE7 is a group A colicin, containing three structural domains with a C-domain bearing non-specific nuclease activity.[21,22] It shares a high level of sequence identity (~70%) with all the E-group colicins as regards T and R-domains, since all the E-group colicins use a common outer membrane cobalamin receptor BtuB for protein import.[3] For unknown reasons, the T-domain of ColE7 also shares sequence identity (21%) with the TR-domain of the group B colicin B (see the sequence alignment in Figure 1), which uses a different receptor and translocation system compared to those of ColE7 for cellular import. We prepared a full-length and an N-terminal tail deleted ColE7 to measure their cytotoxicity to *E. coli*. The ColE7 T-domain and the N-terminal tail deleted T-domain were also purified to test their ability to interact with TolB in co-immunoprecipitation experiments. Moreover, the crystal structure of the N-terminal tail deleted ColE7

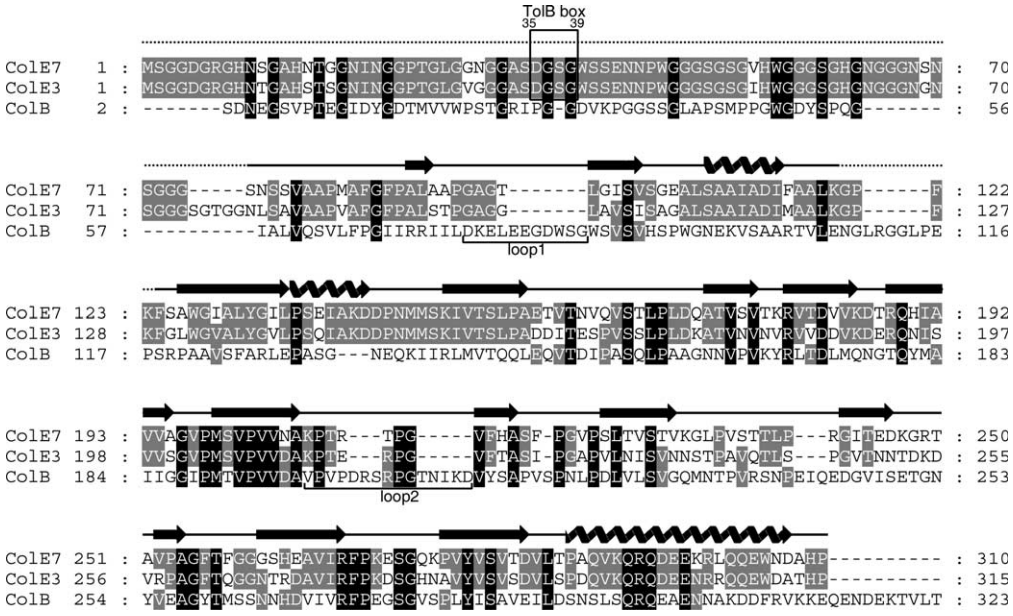

Figure 1. Sequence alignment for the T-domain (translocation domain) in ColE7 and ColE3, and the TR-domain (translocation and receptor-binding domain) in ColB. The residues conserved in all three colicins are shaded in black and these conserved in two colicins are shaded in grey. The TolB box in ColE7 and E3 is marked in the Figure. The secondary structure elements derived from the crystal structure of ColE7 T-domain are displayed on the top row of the sequence.

T-domain was determined at the high resolution of 1.7 Å by the multi-wavelength anomalous dispersion (MAD) method and the structure was compared with the T-domain in ColE3 and the TR-domain in ColB. Our *in vivo* and *in vitro* results confirm that the N-terminal tail of ColE7 is required both for its direct interactions with TolB and for its cytotoxicity. The structural resemblance observed between group A and B colicins indicates that a related translocation step is likely involved for these proteins during cellular import.

Results

ColE7 without the N-terminal tail cannot kill *E. coli*

It was shown that the TolB box with a sequence of 35-DGSGW-39 is of critical importance for cell killing and the interaction between ColE9 and TolB by site-directed mutagenesis and yeast two-hybrid system assays.[11] To find out if the N-terminal tail of ColE7 is also important for cell killing, we purified full-length and N-terminal tail deleted ColE7/Im7 complexes, respectively (ColE7: residues 63–576), and used them in cell killing activity assays. The wild-type ColE7/Im7 spotted on a disk of filter-paper placed in an agar plate seeded with *E. coli* M15 strains killed *E. coli* cells, resulting in a clear region around the filter-paper (see Figure 2). However, the N-terminal tail deleted ColE7/Im7 complex did not kill bacterial cells indicating the N-terminal tail for ColE7 is necessary for its cytotoxicity.

ColE7 T-domain interacts with TolB

Since the *in vitro* binding between TolB and ColE7 has not been reported, we further assayed the direct interactions between TolB and the ColE7 T-domain by co-immunoprecipitation. His-tagged ColE7 T-domain (residues 1–316) or truncated T-domain (residues 63–316) was expressed and purified from *E. coli*. These two proteins were then incubated with antibodies directed against ColE7 T-domain and protein G Sepharose, respectively. After adding the purified TolB to the antibody mixture, we found that TolB precipitated only the ColE7 T-domain but not the truncated T-domain (see Figure 3). This result indicates that the N-terminal tail (residues 1–62) of ColE7 is required for the interactions between its T-domain and TolB. This result is in line with previous data showing that the TolB box located between residues 35 and 39 in nuclease colicins is of critical importance for interactions with TolB.[11]

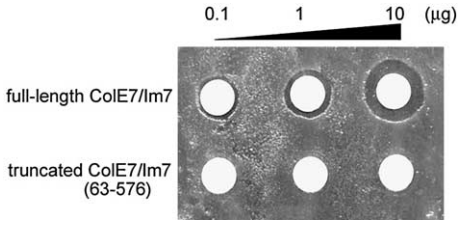

Figure 2. *In vivo* cell death assays for wild-type and N-terminal tail deleted ColE7/Im7. A drop of 20 μl of wild-type or N-terminal tail deleted ColE7/Im7 (0.1–10 μg/ml) was spotted onto a disk of filter-paper in an LB agar plate seeded with *E. coli* M15 strain. Plates were then incubated overnight at 37 °C. The wild-type ColE7/Im7 killed bacteria and generated a clear circle surrounding the spotted site. The deleted ColE7 mutant (residues 63–576) gave no clear zone, showing that it had lost its cell killing activity.

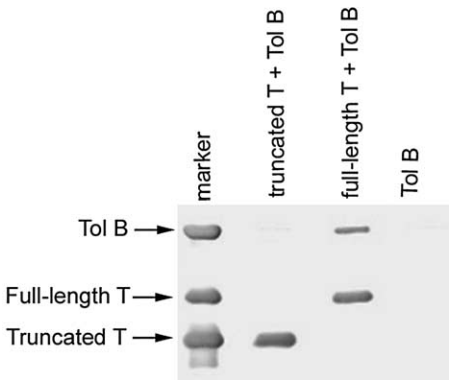

Figure 3. Co-immunoprecipitation of ColE7 T-domain and TolB. Full-length His-tagged ColE7 T-domain (10 μg) and truncated T domain (10 μg) was incubated with anti-T-domain antibodies and an excess of protein G Sepharose, respectively, at 4 °C overnight. His-tagged TolB (10 μg) was then added into the suspension and the protein complexes associated with protein G Sepharose were washed, denatured and separated by SDS-PAGE and immunoblotted with anti-His tag antibodies. TolB co-precipitated only with the T-domain but not with the truncated T-domain (two central lanes). The left lane shows the markers for the His-tagged TolB, T-domain and truncated T-domain. The right lane is the negative control in which TolB was added to the mixture of anti-T-domain antibodies and protein G Sepharose in the absence of the ColE7 T-domain.

Structure determination and overall structure of the ColE7 T-domain

The crystal structure of the ColE7 T-domain could provide useful information for further understanding the interactions between ColE7 and Tol proteins during translocation. Previous NMR studies had shown that the N-terminal 83 residues of ColE9 are largely unstructured and highly flexible,[23] and are perturbed by the binding to TolB.[24] We failed to crystallize the ColE7 T-domain, probably due to disorder in the N-terminal region. But the truncated

Table 1. Data collection, phasing and refinement statistics of ColE7 T-domain

A. *Diffraction data statistics*			
Space group	$P3_121$		
Cell constants (Å and deg.)	$a=59.04$, $b=59.04$, $c=132.18$ $\alpha=90$, $\beta=90$, $\gamma=120$		
Wavelength (Å)	λ_1, 0.9795	λ_2, 0.9797	λ_3, 0.9805
Resolution range (Å)	40–1.7	40–1.7	40–1.7
Observed reflections	315,209	304,670	288,166
Unique reflections	29,971	29,940	29,879
Completeness (%)[a]	99.3 (100.0)	99.1 (100.0)	98.8 (100.0)
$\langle I\rangle/\langle\sigma I\rangle$[a]	68.8 (11.8)	67.2 (12.3)	64.9 (13.1)
R_{merge} (%)[a,b]	8.5 (27.2)	6.3 (24.7)	6.2 (22.9)
B. *Phasing statistics*			
Number of Se sites	4	4	4
Phasing power (centric/acentric)	3.54/3.47	3.61/3.52	2.18/2.15
Figure of merit (centric/acentric)		0.75/0.70	
C. *Refinement statistics*			
Resolution range (Å)			40–1.7
Reflections (working/test)			26,591/2959
R_{work}/R_{free} (%)			18.1/20.9
Number of atoms (protein/water)			1704/303
Average *B*-factor (Å^2) (protein/water)			21.4/33.8
RMS deviations (bond length (Å)/bond angle (degree))			0.015/1.759

[a] The numbers in parentheses are for the last shell in the resolution range of 1.76–1.70 Å.
[b] $R_{merge}=\sum_h\sum_i|I_{h,i}-\langle I_h\rangle|/\sum_h\sum_i I_{h,i}$where $\langle I_h\rangle$ is the mean intensity of i observations for a given reflection h.

T-domain of ColE7 (residues 60–316) without the flexible glycine-rich N-terminal tail was crystallized successfully in a trigonal space group $P3_121$ by the hanging-drop, vapor-diffusion method. The structure was first solved by the molecular replacement method, which gave a possible rotational and translational solution using the T-domain in ColE3 (PDB entry, 1JCH) as the search model. However, the structure could not be refined properly possibly due to the atomic structural differences between ColE3 and ColE7 being large. The selenomethionine (Se-Met)-labeled truncated T-domain was then produced and crystallized. Three sets of X-ray diffraction data were collected using synchrotron radiation at different wavelengths at beamline NW12 in the KEK Photon Factory, Tsukuba, Japan. The structure was solved by the MAD method using anomalous signals from four Se-atoms. The final structural model of the ColE7 T-domain, excluding the flexible N and C-terminal tails, contained residues 75–119 and 124–310 with good geometries and refinement statistics. The diffraction and refinement statistics are listed in Table 1.

The overall fold of the T-domain is shown in Figure 4(a) and the secondary structural elements are displayed on the top row of the ColE7 sequence in Figure 1. The T-domain folds mainly in three layers of β-sheets (shown in different colors in Figure 4(a)) with three helices flanking the sides. The central β-sheet (in red) contains more hydrophobic residues sandwiched between two other β-sheets. The electron density of the N-terminal end of the constructed T-domain including the first five residues from cloning and residues 60–74 of ColE7 was not observed. The first 83 residues in the crystal structure of ColE3 were also not observed,[17] indicating the intrinsic flexibility of this N-terminal tail in group A colicins.

Structural comparison of the T-domain of colicins E7, E3 and B

The crystal structure of ColE3 in complex with Im3 is shown in Figure 4(b). The immunity protein Im3, which inhibits the RNase activity of ColE3, is bound to the C-domain (RNase domain) and located between the C and R-domains.[17] ColE7 shares high sequence identity with ColE3 in T and R-domains (73.8%), but the C-domains of the two colicins are completely different, one with RNase and one with nuclease activity. The structure of ColE7 T-domain was superimposed with that of ColE3 and the average root-mean-squared difference (rmsd) was 1.28 Å over 190 C^α atoms (see Figures 4(a) and 5(a)). The two T-domains bear a similar fold, the only difference being that the high-resolution structure of the ColE7 T-domain (1.7 Å for ColE7 *versus* 3.0 Å for ColE3) reveals more ordered secondary structures. This result confirms that E-group colicins E2 to E9 share a similar folded T-domain.

The crystal structure of the ColE7 T-domain was submitted to structural homology searching servers: DALI[25] and MATRAS.[26] As a result, in addition to ColE3, ColB was identified with a high matching score. The crystal structure of the ColE7 T-domain was therefore superimposed with the TR-domain of ColB, giving an average rmsd value of 1.26 Å over 167 C^α atoms (see Figures 4(c) and 5(b)). In the crystal structure of ColB, a fragment of its N-terminal tail from Glu11 to Arg25 containing the TonB box sequence of 17-DTMVV-21 is ordered and present. This N-terminal peptide is folded back and interacts with Arg170, Tyr259 and Met261 in the TR-domain. Three aligned residues (see Figure 1) in ColE7 are Arg179, Phe256 and Phe258, in which Arg179 is

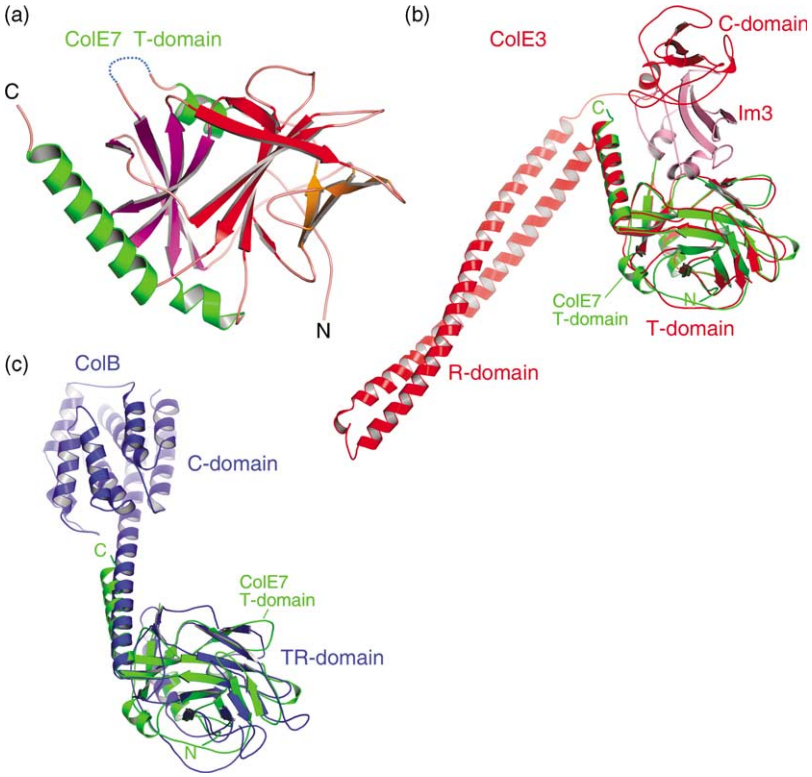

Figure 4. Structures of the ColE7 T-domain and its superposition onto ColE3 and ColB. (a) The crystal structure of the ColE7 T-domain contains three central layers of β-sheets (in magenta, red and gold) flanked by three α-helices. The N-terminal end (residues 60–75) and a loop region from residues 120–123 (displayed in a broken line) are disordered and not seen in the structure. (b) The crystal structure of ColE3 (PDB entry, 1JCH) contains three structural domains assembled in a Y shape: T-domain, R-domain and C-domain (displayed in red). The immunity protein Im3 is displayed in pink. The T-domain of ColE7 (in green) is superimposed on that of ColE3. (c) The crystal structure of ColB (PDB entry, 1RH1), displayed in blue, is folded in a dumbbell shape, with a pore-forming C-domain located at one side of a long α-helix and an intertwined TR-domain located at the other side. The T-domain of ColE7 (in green) is superimposed on the TR-domain of ColB.

identical with Arg270 in ColB; Phe256 is an aromatic residue homologous to Tyr259 in ColB. This result suggests that the N-terminal tail of ColE7 might be folded in a way similar to that of ColB.

Discussion

Plausible protein–protein interacting surface in ColE7 T-domain

The only nuclease colicin crystal structures currently available are the C-domains of colicin E7[22] and E9[27] in complex with their respective immunity proteins. Now that we have solved the crystal structure of the ColE7 T-domain, it might be possible to build a structural model of ColE7 based on the structure of ColE3. Using a protein docking program ZDOCK,[28] we successfully docked the two crystal structures of ColE7 C-domain/Im7 (PDB entry, 1MZ8) and T-domain together. A protein–protein interacting interface with complementary polarity and shape was identified with a buried area of 1807 Å^2 between the T-domain and C-domain/Im7, as calculated in the protein–protein interaction server.[29] A structural model of full-length ColE7/Im7 was then constructed by adding the ColE3 R-domain (PDB entry, 1JCH) to the docked ColE7 T and C-domain. This model was further refined to minimize potential energy with the final structure and is shown in Figure 6.

From mutagenesis studies, it is known that several group A colicins, such as colicins A and E1–E9, require two outer membrane protein (Omp) receptors for import and cytotoxicity.[3] The initial step of colicin uptake involves the interactions of the R-domain with a specific receptor and denaturation of colicin on the cell surface.[30] For E-group colicins, it is suggested that the tip of the coiled-coil R-domain binds the cork region of the β-barrel structure of BtuB, as evident by the crystal structure of ColE3 R-domain in complex with BtuB.[31] It is further suggested that the T-domain may be involved in interactions with a second transporter, such as OmpF in the cases for colicins A and E2–E9.[3] In this translocation model, the flexible N-terminal tail in the T-domain of group A colicins interact with the second transporter to initiate the so-called "threading" of colicins through the pore.[1,3] TolB in the periplasm interacts with the outer-membrane peptidoglycan-associated lipoprotein Pal,[29] hence TolB is located close to the outer membrane. Thus, the interactions between TolB and colicins may further pull colicins into the periplasm.[7] Our results confirm that the direct interactions between the ColE7 N-terminal tail and TolB and the requirement of the N-terminal tail of T-domain account for colicin's cytotoxicity. However, at present it is not certain that the loss of the cytotoxicity for the N-terminal tail deleted ColE7 is due to its deficiency in interactions with TolB or with other interacting partners. Except for some mutagenesis studies showing that OmpF$^-$

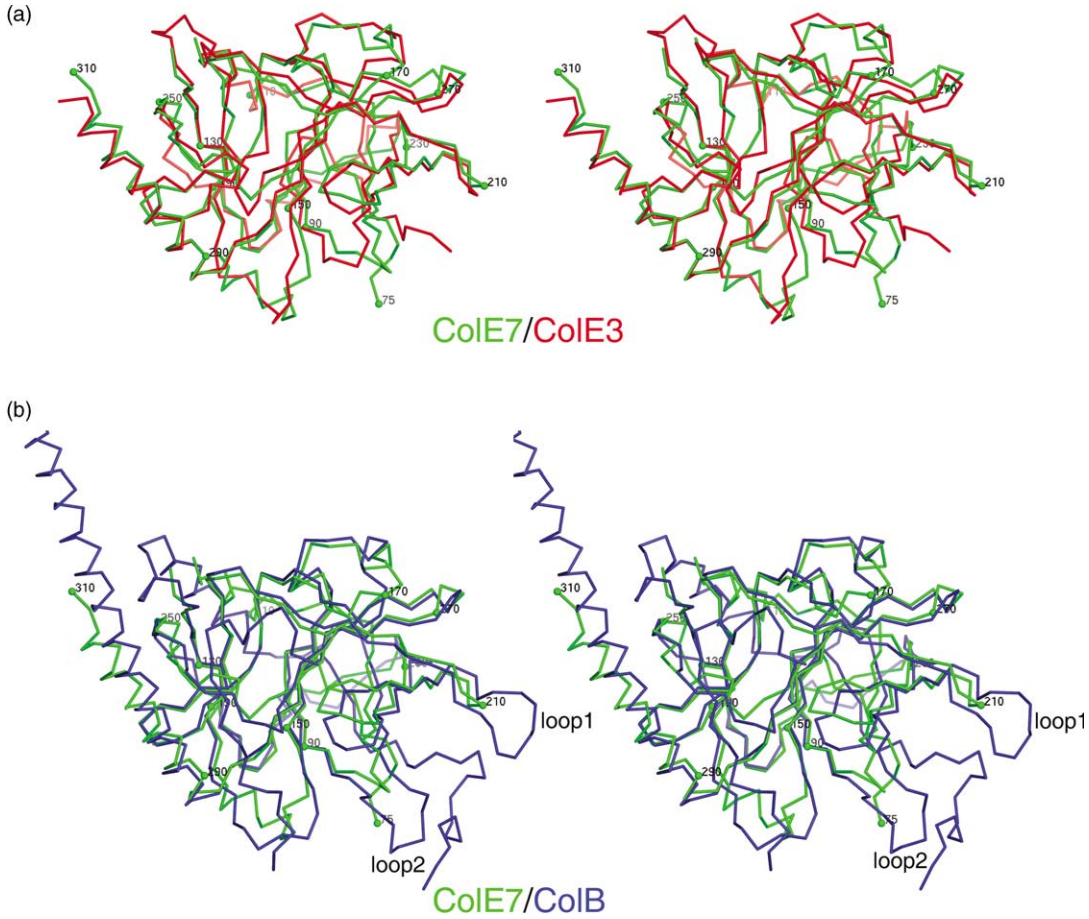

Figure 5. The superposition of the C^{α} backbones of the T-domain in ColE7/ColE3 and ColE7/ColB. (a) The T-domain of ColE7 matches well with that of ColE3 with an average rmsd value of 1.28 Å for 190 C^{α} atoms. (b) The T-domain of ColE7 is superimposed with the TR-domain of ColB with an average rmsd value of 1.26 Å for 167 C^{α} atoms.

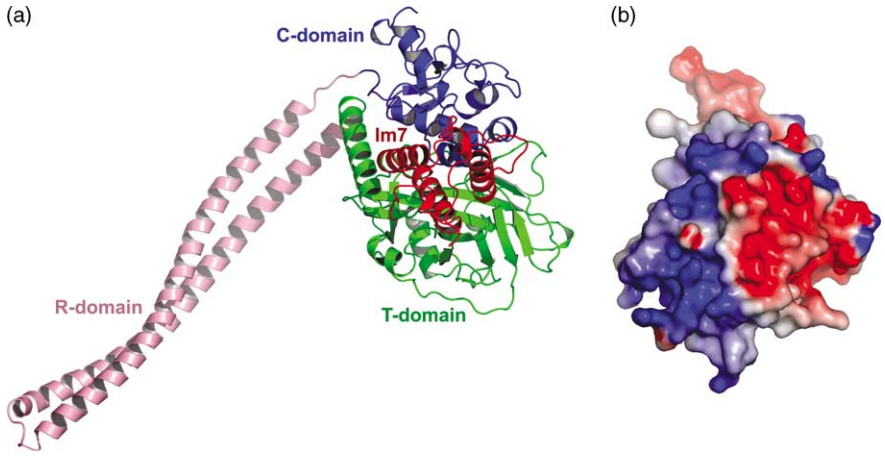

Figure 6. Structural model of ColE7/Im7 complex. (a) The crystal structure of the ColE7 T-domain was docked with the crystal structure of the C-domain/Im7 complex (PDB entry, 1MZ8) by ZDOCK.[28] The full-length ColE7/Im7 model was constructed using the crystal structure of ColE3 (PDB entry, 1JCH) as a template to assemble the R-domain with T/C domains by Modeller 8.1.[41] (b) The molecular surface of the ColE7 T-domain is mapped with electrostatic potential (electropositive charges in blue and negative in red). This view is rotated ~180° vertically from the one in (a) to show the convex surface, which is facing outward, not blocked by the C-domain and Im7. The left region of the T-domain in this view is positively charged and this surface is highly conserved among ColE2, E3, E6, E7 and E9. This conserved basic region is suggested to be tested for a role in protein or membrane interactions during colicin import.

mutants are resistant to E-group colicins,[32] the T-domain of ColE3 (residues 1–285) has been shown to occlude OmpF channels.[33] Moreover, the disordered N-terminal tail in the ColE9 T-domain is involved in recruiting OmpF to the BtuB-ColE9-Im9 translocon in the outer membrane.[34] By comparison of the sequences of all the E-group colicins, which use both BtuB and OmpF for import, including ColE2, E3, E6, E7 and E9, we have found that several regions on the surface of the T-domain are highly conserved. One conserved region facing outward and not buried between different domains in our structural model is more basic than other conserved surfaces (see Figure 6(b)). Since the extra-cellular region of OmpF is acidic,[35] except for the flexible N-terminal tail, this basic conserved region is suggested here to be tested for direct interactions with OmpF or other interacting partners.

Implication for membrane translocation

ColB is a TonB-dependent colicin that recognizes outer membrane protein FebA as its receptor.[36] It was thus surprising to find out that it matched well with the Tol-dependent ColE7, even though a similar topology between ColB and ColE3 has been noticed.[20] The major structural differences between ColE7 and ColB are located in two loops (marked as loop1 and loop2 in Figures 1 and 5(b)) between β-strands, which are much longer and extend outward in ColB. ColB does not contain a distinct R-domain responsible for its interactions with receptor FebA, therefore it is not clear how ColB binds FebA to initiate its cellular import. Based on the structural comparison between ColE7 and ColB, we suggest that the two long loops, loop1 (Asp75–Gly85) and loop2 (Val198–Asp212), identified in ColB facing the same side and containing a number of charged residues, are candidate sites to be examined further for receptor binding.

Finally, we show that the T-domains in ColE7, ColE3 and ColB share a structural resemblance. It is unclear why the group A colicins, ColE7 and ColE3, have a folded T-domain similar to that of a group B colcin (ColB), since the two groups of colicins interact with different protein partners during translocation. The structural and sequence homology may have evolved from the same origin. Alternatively, group A and group B colicins may share similar mechanisms or interacting partners during cellular import. For example, lipids have been suggested to be possible translocation sites for Gram-negative bacteria[1] and both group A and B colicins come across lipopolysaccharide (LPS) in the outer membrane. The structural conservation of the T-domain among Tol-dependent and TonB-dependent colicins implies a mechanistic connection during cellular import for group A and B colicins, leading to an interesting subject worthy of future examination.

Methods

Protein expression and purification

T-domain and truncated T-domain

The gene encoding ColE7 T-domain (residues 1–316) and truncated T-domain (residues 60–316) was amplified by PCR from the plasmid encoding the full length of ColE7/Im7 in a pQE-70 vector prepared previously.[37] It was then cloned into the SphI-BglII site of the pQE-70 vector (Qiagen). The two expression vectors were transformed, respectively, into *E. coli* M15 cells, cultured using LB medium (100 μg/ml of ampicilin) to an A_{600} of ~0.7 and then the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h. The crude cell lysate suspended in buffer A (50 mM phosphate buffer (pH 8), 300 mM NaCl) was loaded onto a Ni-NTA column (Qiagen, Germany). The His-tagged T-domain was eluted by a gradient of buffer A containing 500 mM imidasole at pH 7. The eluent was then dialyzed in 50 mM phosphate buffer (pH 7) and applied onto a Sepharose-SP column (HiTrap SP, Pharmacia) equilibrated with the same buffer. The T-domain and truncated T-domain were eluted with a NaCl gradient and the collected fractions were further dialyzed in 50 mM Tris–HCl (pH 7.6) and 5% (v/v) glycerol before it was concentrated to 15 mg/ml and 12 mg/ml, respectively.

Se-Met-labeled T-domain

The Se-Met-labeled truncated T-domain was expressed using the same expression vector of pQE-70, and transformed into *E. coli* BL834 (DE3) competent cells. The initial small-scale culture was incubated at 37 °C in LB medium (100 μg/ml of ampicilin) overnight, similar to the wild-type protein. The bacterial solution was then transferred into large-scale minimal medium M10, containing 80 mg/l of all amino acids, where methionine was substituted with seleno-L-methionine.[38] Protein overexpression was induced by addition of 1 mM IPTG at an A_{600} of ~0.7 and incubation continued for further a 15 h at 30 °C. The Se-Met-labeled truncated T-domain was purified by a two-step chromatography method similar to that of the non-labeled protein. The purity of the two proteins was checked by SDS-PAGE and mass spectroscopy. The recombinant truncated T-domain contained the residues 60–316 of ColE7, an N-terminal tail of five residues from cloning (MRGSR) and a C-terminal histidine tag (-RSHHHHHH). A molecular mass of 28,385 Da was measured for the Se-Met-labeled ColE7 T-domain by mass spectroscopy, closed to the calculated mass of 28,383 Da.

Full-length and truncated ColE7/Im7

The gene encoding truncated ColE7/Im7 (ColE7, residues 60–576; Im7, residues 1–89) was amplified by PCR from the plasmid encoding the full-length ColE7/Im7, and then cloned into the SphI-BglII site of the pQE-70 vector (Qiagen). Similar purification procedures[37] using a Ni-NTA resin affinity column (Qiagen, Germany) followed by a carboxymethyl column (Pharmacia) were used to purify the full-length and truncated ColE7/Im7 complexes.

Cell death assay

Purified full-length or truncated ColE7/Im7 complexes were serially diluted in 20 mM sodium phosphate buffers.

A drop of 20 μl of protein solution was spotted at each dilution onto a disk filter-paper placed in freshly prepared ampicillin LB agar plates seeded with *E. coli* M15 strains. Plates were then incubated overnight at 37 °C. The wild-type ColE7/Im7 killed cells and gave a clear region around the filter-paper (see Figure 2), but the truncated ColE7/Im7 did not kill cells and gave no clear region around the spotted filter-paper.

Co-immunoprecipitation

Purified 10 μg of full-length T domain and 10 μg of truncated T domain were incubated with anti-T domain antibodies and an excess of protein G Sepharose, respectively, at 4 °C overnight in 500 μl of TBS buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl). The pellet was washed three times and re-suspended with the buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM KCl and 2 mM DTT. Afterwards, 10 μg of TolB was added into the suspension, which was then incubated at 4 °C for 4 h in a final volume of 500 μl. The immunoprecipitate was then washed several times with the same buffer before it was denatured and separated by SDS-PAGE, followed by immuno-blotting with anti-His tag antibodies.

Crystallization and X-ray data collection

Crystallization trials were performed by the hanging-drop, vapor-diffusion method at 5 °C. The Se-Met-labeled T-domain was crystallized from a drop containing 0.05 M Tris–HCl (pH 8.5), 0.75 M $(NH_4)_2SO_4$ and 6% glycerol against a reservoir of 0.1 M Tris–HCl (pH 8.5), 1.5 M $(NH_4)_2SO_4$ and 12% glycerol. Large single crystals appearing within two weeks were flash-cooled in liquid nitrogen without additional cryo-protecting solutions before data collection.

After a wavelength scan for selenium absorption in the Se-Met/T-domain crystal, three data sets were collected at −150 °C by a Quantum210 CCD detector at beamline NW12 in KEK Photon Factory, Tsukuba, Japan. The SeMet-labeled T-domain crystallized in trigonal space group $P3_121$ with one molecule per asymmetric unit. Diffraction data were processed with HKL2000[39] and diffraction statistics are listed in Table 1.

Structural determination and refinement

The crystal structure of ColE7 T-domain was determined by the MAD method using anomalous signals from Se-Met-labeled protein crystals. The four expected selenium sites were identified and located using an automated Patterson heavy-atom search program in CNS.[40] Positions and occupancies of the four selenium atoms were further refined, giving good occupancies (1.0–1.2), reasonable temperature factors (12 Å^2 to 24 Å^2) and good phasing statistics, as listed in Table 1. At this point, the initial phases were improved by density modification techniques in CNS and the first Fourier map was calculated, clear enough for tracing most of the polypeptide chain. Manual modifications of the model were performed on an SGI Fuel machine using the program Turbo-Frodo. Subsequent structural refinements with CNS were then carried out, including maximal likelihood, simulated annealing protocol and positional and *B*-factor refinement. The first 15 N-terminal residues, including the five residues from cloning (MRGSR) and the residues 60–74, and the last 14 C-terminal residues, including residues 311–316 and the histidine tag (-RSHHHHHH) were not observed in the map due to disorder. The electron density for residues 120–123 was also ill defined, so the final structural model contained residues 75–119, residues 124–310, 303 water molecules, a glycerol molecule and a sulfate ion with a final *R*-factor of 18.1% and *R*-free of 20.9% for 29,550 reflections in the resolution range of 40.0 Å −1.7 Å. The programs PyMOL, DeepView, BioEdit and GeneDoc were used in producing Figures.

Protein docking and homology modeling

The automatic docking system, ZDOCK2.3,[28] was used to perform the protein–protein docking for the T-domain and the C-domain/Im7 (PDB entry, 1MZ8). After assigning the charges by the program mark_sur, Zdock used a fast Fourier transform to calculate the possible binding orientations and evaluated shape complementarity, desolvation energy, and electrostatics in the protein interface. The top 2000 predicted complexes were examined by fitting the docked complex onto the TR-domain of ColE3 (Pdb entry, 1JCH) by superimposition of the two T-domains. Only the complexes whose C-domain's N-terminal end was located within a reasonable distance from the R-domain's C-terminal end were chosen for further examination. The tenth protein complex with a score of 47.25 in Zdock was used to build the final model comprising ColE7 T-domain, R-domain and C-domain in complex with Im7. This model was generated and further optimized with Modeller 8.1.[41]

Protein Data Bank accession codes

Structural coordinates and diffraction structure factors of the ColE7 R-domain have been deposited in the Protein Data Bank with RCSB ID code of rcsb034426 and PDB ID code of 2AXC.

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