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Hierarchical Order of Critical Residues on the Immunity-Determining Region of the Im7 Protein Which Confer Specific Immunity to Its Cognate Colicin

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The directed mutagenesis study of the Im7 protein of colicin E7 revealed that three residues, D31, D35, and E39, located in the loop 1 and helix 2 regions of the protein were critical for initiating the complex formation with its cognate colicin E7. Interestingly, the importance of these three critical residues in conferring specific immunity to its own colicin was exhibited in a hierarchical order, respectively. Moreover, we found that existence of the three critical residues was common among the DNase-type Im proteins. Most likely the three residues of the DNase-type immunity proteins are critical for initiating the unique protein–protein interactions with their cognate colicin. In addition, replacement of the helix 2 of Im7 by the corresponding region of Im8 produced a phenotype of the mutant protein very similar to that of Im8. This result suggests that the DNase-type Im proteins indeed share a "homologous-structural framework" and evolution of the Im proteins may be engendered by minor amino acid changes in this specific immunity-determining region without causing structural alteration of the proteins.

The E-group colicins are plasmid-borne bacteriocins, which exhibit antibiotic-like inhibition of growth of sensitive *Escherichia coli* and closely related coliform bacteria (1). Colicin-producing organisms coexpress a specific immunity protein, which forms a tight, stoichiometric complex with the C-terminal toxic domain of colicin thus neutralizing its toxicity in the host. E-group colicins fall into three cytotoxic classes, including pore-forming agents such as colicin E1 (2), RNase such as colicin E3 (3) and E6 (4), and DNase such as colicin E2 (5), E7 (6), E8 (7) and E9 (6).

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The DNase-type colicins have almost identical amino acid sequences in their translocation and receptor recognition domains. Otherwise, the C-terminal endonuclease domains (T2A domain) are approximately 80% identical and sequences of their corresponding immunity proteins are 60–70% identical (6, 8). However, despite the high level of sequence identity, an immunity protein can only completely protect a cell from the action of its own cognate colicin at the normal level of expression implicating that each immunity protein neutralizes exclusively its specific colicin endonuclease domain (9). The crystal structure of the Im7 protein shows that several exposed acidic residues located at loop 1, helix 1, helix 2, and loop 2 regions are possibly involved in specific binding to the basic endonuclease domain of colicin (10). NMR chemical shift perturbation studies (11) have demonstrated that in addition to helix 2, helix 3 which is homologous in the DNase-type immunity family, is important for general binding.

In the present study, we demonstrate that the existence of three critical residues located at the loop 1 and helix 2 regions of the ImE7 protein are specifically responsible for the initiation of ColE-Im complex formation. However, helix 2 of the protein as a whole determines the specificity of inhibitory effect (immunity) against its cognate colicin. For some extent, these results also provide invaluable information for understanding the evolution of the DNase-type immunity proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmid pUC18 was used as the cloning vector. ColE7-K317, ColE8-J and ColE2 were used as templates for cloning *ceiE7* and *ceiE8* and *ceiE2* genes, respectively. *E. coli* JM101 was used as the host for recombinant plasmids.

Site-directed mutagenesis mediated by polymerase chain reaction. The strategy and detailed procedure for PCR-mediated, oligonucleotide-directed site specific mutagenesis was as previously

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TABLE 1

Oligonucleotide Primer Sequences for the Construction of Mutant *ceiE7* Gene in Various Recombinant Plasmids

Plasmid constructed	Oligonucleotide primer sequences	Position on *ceiE7*[1]	Template used
pIE7-1	F: 5′-GCAACTAATGATGTGTTAGATG-3′	+134–+155	pIE7
	R: 5′-AACACATCATTAGTTGCAG-3′	+150–+132	
pIE7-2	F: 5′-CAACTGATAATGTGTTAGATG-3′	+135–+155	pIE7
	R: 5′-ATCTAACACATTATCAGTTGC-3′	+154–+134	
pIE7-5	F: 5′-TGTGTTAAATGTGTTACTC-3′	+145–+163	pIE7
	R: 5′-GTAACACATTTAACACATCATC-3′	+161–+140	
pIE7-9	F: 5′-GTTACTCCAACACTTTG-3′	+157–+173	pIE7
	R: 5′-CAAAGTGTTGGAGTAAC-3′	+173–+157	
pIE7-59	F: 5′-GTTACTCCAACACTTTG-3′	+157–+173	pIE7-5
	R: 5′-CAAAGTGTTGGAGTAAC-3′	+173–+157	
pIE7-259	F: 5′-GCAACTGATAATGTGTTAAATGTG-3′	+134–+157	pIE7-9
	R: 5′-CACATTTAACACATTATCAGTTGC-3′	+157–+134	
pIE7-2590	F: 5′-TATCCTAGTAATAATAGAGACG-3′	+215–+236	pIE7-259
	R: 5′-TATCGTCTCTATTATTACTAGG-3′	+239–+218	
pIE7M	F: 5′-GCAACTAATAATGTGTTAAATG-3′	+134–+155	pIE7-2590
	R: 5′-AACACATTATTAGTTGCAG-3′	+150–+132	
pIE7-1590	F: 5′-GCAACTAATGATGTGTTAAATG-3′	+134–+155	pIE7M
	R: 5′-AACACATCATTAGTTGCAG-3′	+150–+132	
pIE7-159	F: 5′-CAACTAATGATGTGTTAAATGTG-3′	+135–+157	pIE7-9
	R: 5′-ATTTAACACATCATTAGTTGCAG-3′	+154–+132	
pIE7-125	F: 5′-CAACTAATAATGTGTTAAATGTG-3′	+135–+157	pIE7-5
	R: 5′-ATTTAACACATTATTAGTTGCAG-3′	+154–+132	
pIE7-129	F: 5′-CAACTAATAATGTGTTAAATGTG-3′	+135–+157	pIE7-9
	R: 5′-ATTTAACACATTATTAGTTGCAG-3′	+154–+132	
pIE7-0	F: 5′-TATCCTAGTAATAATAGAGACG-3′	+215–+236	pIE7
	R: 5′-TATCGTCTCTATTATTACTAGG-3′	+239–+218	
pIE7-150	F: 5′-CAACTAATGATGTGTTAAATGTG-3′	+135–+157	pIE7-0
	R: 5′-ATTTAACACATCATTAGTTGCAG-3′	+154–+132	
ExtF	F: 5′-GTAAAACGACGGCCAGT-3′	pUC forward primer[2]	
ExtR	R: 5′-AACAGCTATGACCATG-3′	pUC reverse primer[2]	

Note. The mismatched bases are underlined.
[1] Nucleotide sequence of *ceiE7* gene has previously been reported by Chak *et al.* (6).
[2] The universal primers flank on both sides of the multiple cloning site of pUC18.

described (12, 13). A pair of universal external oligonucleotide primers, ExtF and ExtR flanking the cloning site of pUC18 for PCR is listed in Table 1. The carboxyl groups of the negatively charged residues in the surface of the putative specific immunity-determining region of the Im7 proteins (10) were changed to amide groups. The amino acid sequences alignment of Im7, Im8, Im2, Im9, and the hybrid protein ImMi are listed in Fig. 1. All mutants generated are listed in Table 2, Figs. 2 to 3, respectively.

In order to test the role of the loop 1–helix 2 region of immunity protein for specific immunity, we modified this region of Im7 toward Im8 by site-directed mutagenesis. Based on the sequence of Im7, we deleted V27 and A29 in order to keep the length of the loop 1 of the mutant the same as that of Im8 (Fig. 1). In addition, the following residues of Im7 were mutated in helix 2: D31E, D32K, V33K, L34Q, V36D, L37N, V42I, K43S, and I44V. The resulting chimeric protein was designated as ImMi. Sequences of primers for the construction of ImMi are listed in Table 1. The detailed procedures of the three-step site-directed mutagenesis for the construction of ImMi are shown as follows:

Step 1. The result of first step site-directed mutagenesis caused deletion of V27 and A29 in the loop 1 region of Im7. In addition, D31E, D32K, V33K, and L34Q mutations were created in the helix 2 region.

D31E D32KV33K L34Q

Forward: 5′-AGAAT-CGT-ACTGAGAAGAAGCAAGATG-3′

(+123 to +155 of *ceiE7*)

Reverse: 5′-CTTCTCAGT-AGC-ATTCTCTTTTTCAATTTC-3′

(+145 to +110 of *ceiE7*)

Step 2. The resultant mutant DNA from first step was used as template for the second step of mutagenesis. As a result, V36D and L37N mutations were created in the helix 2 region.

V36D L37N

Forward: 5′-GAAGAAGCAAGATGATAACCTCGAACAC-3′

(+142 to +169 of *ceiE7*)

Reverse: 5′-TCGAGGTTATCATCTTGCTTCTTCTCAG-3′

(+165 to +142 of *ceiE7*)

Step 3. The resultant mutant DNA from second step was used as template for the third step of mutagenesis. As a result, V42I, K43S and I44V mutations were created in the helix 2 region.

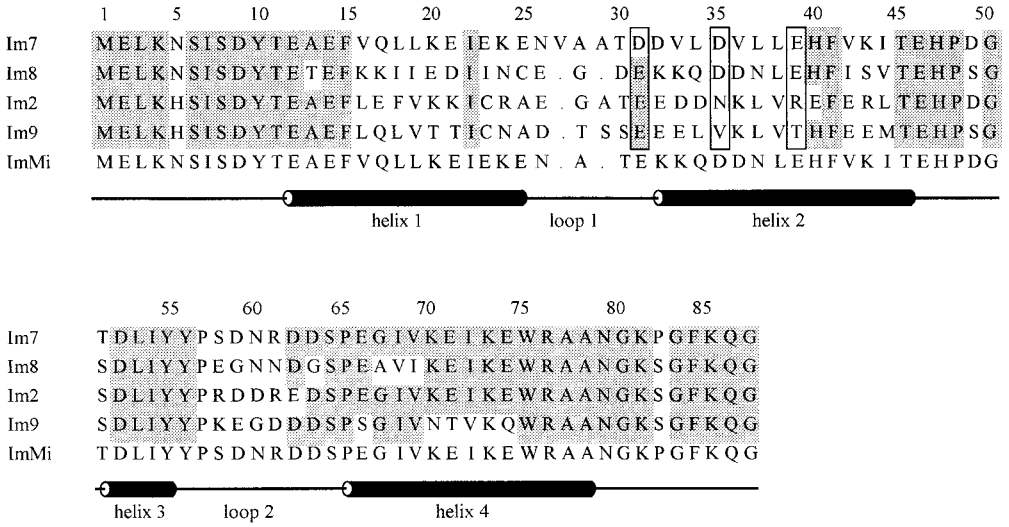

FIG. 1. Amino acid sequences and sequences alignment of the four DNase-type immunity proteins and ImMi proteins. ImMi is a hybrid protein derived from replacement of helix 2 of Im7 by the same region from Im8. The numbers refer to the position of residues of the Im7 protein. The location of four helices and loops are indicated below the sequences. Construction of the mutant protein ImMi is shown in Fig. 4. The position of the three critical residues for conferring specific immunity is shown in boxes.

V42I K43S I44V

Forward: 5′-CGAACACTTTATAAGTGTTACTGAGC-3′

(+163 to +188 of *ceiE7*)

Reverse: 5′-GCTCAGTAACACTTATAAAGTGTTCG-3′

(+188 to +163 of *ceiE7*)

The mismatched nucleotides are underlined. Dash indicates deletion of an amino acid residue in the corresponding positions, respectively. The positions of the mutated residues are indicated. The positions of the oligonucleotide primers on *ceiE7* gene are also shown.

Protein purification and CD spectroscopy. The detailed purification methods for both wild-type and mutant Im proteins are previously described (14, 15). Protein purity was examined by SDS-PAGE. The quantity of purified proteins was checked by DC protein assay (Bio-Rad). The secondary structure of the mutant proteins was examined by CD spectroscopy as described by Chak *et al.* (1998).

Preparation of colicin and in vivo immunity test. These methods are the same as previously described (13).

RESULTS

Identification of critical residues conferring specific immunity for Im7. The negatively charged residues D31, D32, D35, E39, and D59 in the loop 1–helix 2–loop 2 region are highly exposed to solvent and are probably involved in specific interactions with colicins (Fig. 1, 10). Thus they were selected as candidate residues for the analysis of the recognition between immunity and colicin proteins.

Site-directed mutagenesis was used to change the carboxyl group of these acidic residues either individually or in multiple combinations to the amide group (see Materials and Methods). The phenotype of mutants against the colicin E7 is shown in Table 2. Substitution of any of the five residues either with alanine (data not shown) or their amide group (Table 2) individually did not diminish the immunity towards colicin E7. Moreover, mutant containing multiple mutations, D32N + D35N + E39Q + D59N still conferred immunity to colicin E7 (Table 2). Most interestingly, reduction of the immunity phenotype of the mutant was observed only if D31N was included in the Im protein with multiple mutations (Table 2). In immunity tests, cells containing pIE7-159 (D31N + D35N + E39Q), pIE7-125 (D31N + D32N + D35N), and pIE7-129 (D31N + D32N + E39Q) produced clear zones in diminishing order (Fig. 2). Furthermore, cells containing pIE7-150 (D31N + D35N + D59N) produced the smallest and a turbid zone in immunity testing. Thus these results would indicate that D31 appears to be the most critical residue, and that D32 and D59 are the two least important residues for conferring specific immunity. Based on the sizes of inhibitory zones produced in immunity tests, the deduced hierarchical order of the residues for conferring specific immunity against its cognate ColE7 is D31 > D35 > E39. The intervals of these three critical residues are separated by 4 amino acids (Fig. 1), therefore, these three critical acidic residues are exposed to the same side of the surface of the helix 2 of the Im7 protein (10).

The critical residues conferring specific immunity for other DNase-type Im proteins. To further investigate the existence of the three critical residues in other DNase-type Im proteins, an experiment was set up to disrupt the specific immunity of Im8 and Im2 proteins

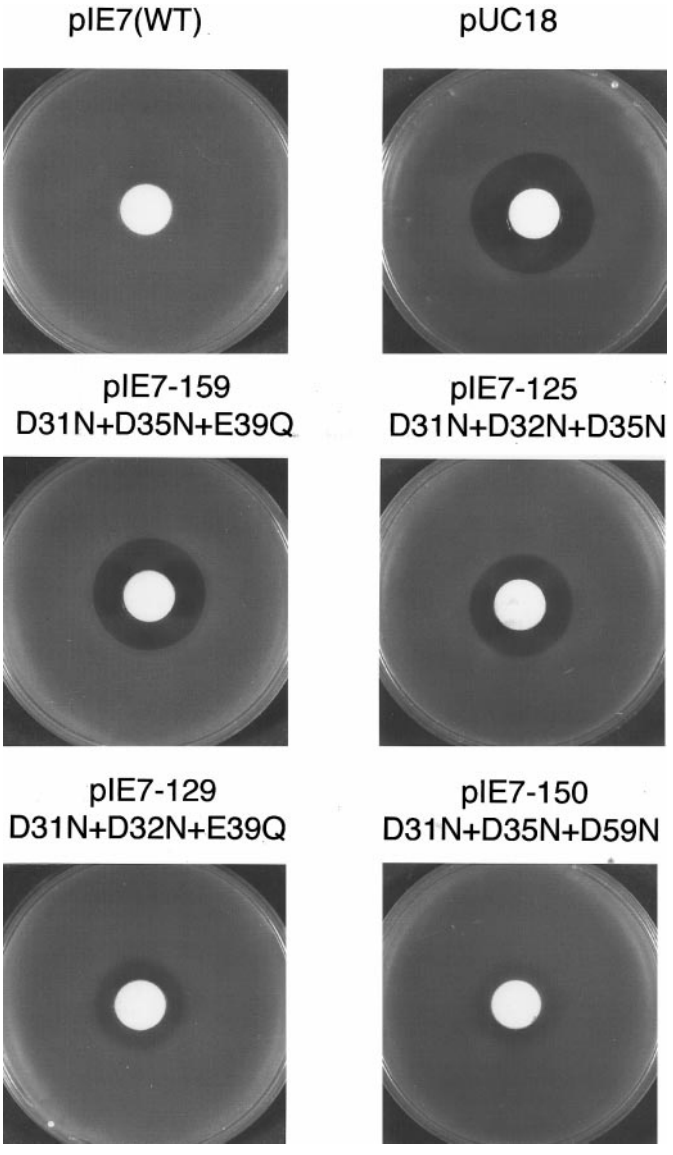

FIG. 2. Identification of the importance of critical residues for conferring specific immunity in loop 1–helix 2 region of Im7 by *in vivo* immunity assay. An aliquot (20 μl) of colicin E7 were spotted onto a blank disk on a LA plate seeded with cells harboring various recombinant plasmids. *E. coli* cells harboring a wild-type *ceiE7* gene on a recombinant plasmid (pIE7) as a positive control. *E. coli* cells containing pUC18 cloning vector as a negative control. pIE-159 (D31N + D35N + E39Q), pIE-125 (D31N + D32N+D35N), pIE-129 (D31E + D32N + E39Q), and pIE-150 (D31N + D35N + D59N) are the four recombinant plasmids containing multiple combinations of mutations along the loop 1–helix 2–loop 3 region of the Im7 protein. The sequences and the positions of oligonucleotide primers on *ceiE7* gene are listed in Table 1.

based on what we had found for the Im7 protein. The Im8 protein is two residues shorter than that of the Im7 protein. Thus the residues E29, D33 and E37 of the Im8 protein are equivalent to those of the three critical residues for specific immunity of the Im7 protein (Fig. 1). The results of mutagenic studies showed that a single point mutation of Im8, either E29Q, D33N or E37Q retained the Im8 phenotype (data not shown). In contrast, cells containing pIE8M (E29Q + D33N + E37Q) in the immunity assay produced a clear inhibition zone similar to that of the cells harboring pUC18 (Fig. 3) suggesting that E29, D33 and E37 of the Im8 are the three critical residues conferring specific immunity to its cognate ColE8. Similarly, mutation of Im2 at the corresponding regions, E30Q + N34A + R38A (Im2 is one residue shorter than Im7, Fig. 1), lost inhibitory effect to its cognate ColE2 (data not shown). An earlier finding showed that changing V34 to D (equivalent D33 for Im8 and D35 for Im7, Fig. 1) of Im9 gained some cross-reactivity towards colicin E8 (16). Thus all data imply that the three critical residues for conferring specific immunity are common in DNase-type immunity proteins.

Helix 2 as a whole plays a crucial role in conferring specificity for immunity proteins. The amino acid sequence alignment of the immunity proteins indicated that loop 1 and helix 2 are the most variable regions among the tested Im proteins (Fig. 1). In this work we show that loop 1–helix 2 is the common specific immunity-determining region of the DNase-type immunity proteins. Thus, it may be possible to alter the immunity phenotype from one type to another by simply swapping the helix 2 among the immunity proteins. In order to test this hypothesis, we attempted to change the phenotype of Im7 to Im8 by swapping the helix 2 of the Im7 with the corresponding region of the Im8 protein.

The resultant recombinant plasmid, pImMi was constructed (see Materials and Methods, Fig. 4) and the immunity assay showed that the phenotype of cells harboring pImMi did not confer immunity against the ColE7 but exerted partial immunity against ColE8 (Fig. 4). Cells containing wild-type ColE8 plasmids confer partial immunity to colicin E7 (Fig. 4). Thus the result indicated that phenotype of cells harboring pImMi had been converted from Im7 toward Im8. In conclusion, the specific spatial arrangement of all residues in helix 2 region are crucial for conferring the overall specific immunity of an Im protein.

CD spectroscopy of wild-type and mutant Im7 proteins. The negatively charged residues D31, D32, D35, and E39 located in the loop 1–helix 2 region are highly exposed to solvent (10). Therefore, it can be supposed that replacement of the carboxyl group of these acidic residues to amide group by site-directed mutagenesis could possibly maintain the original conformation of the mutant Im protein. Nevertheless, CD spectroscopy was used to investigate the possible change of secondary structure of the mutant Im proteins. The far-UV spectra of the CD measurement of wild-type and mutant Im7 proteins were very similar

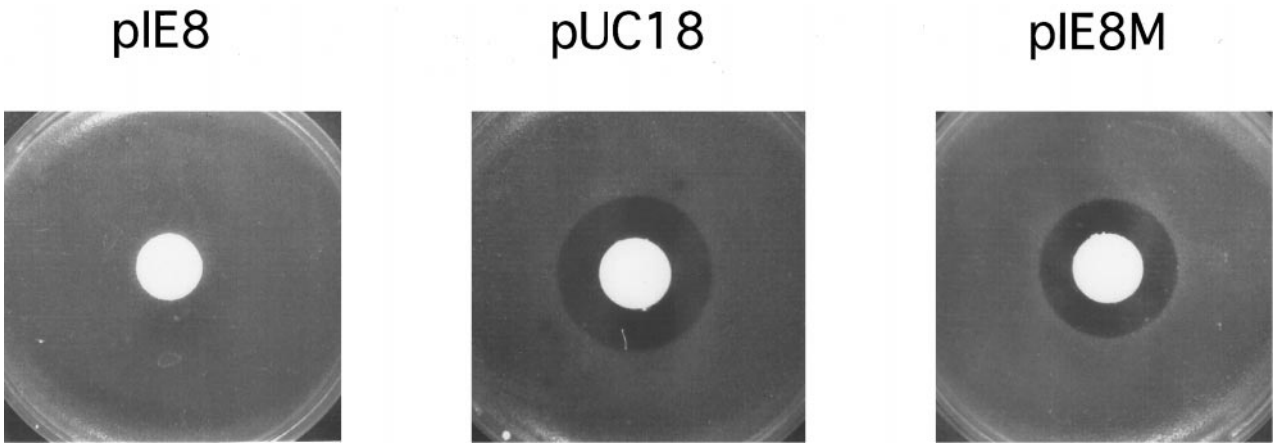

FIG. 3. Identification of critical residues for conferring specific immunity of the Im8 protein by *in vivo* immunity assay. *E. coli* (pIE8) cells harboring plasmid with wild-type *ceiE8* gene, served as positive control. *E. coli* (pIE8M) are the cells harboring the plasmid with the mutated *ceiE8* gene. Three mutations, E29Q + D33N + E37Q, were generated in the region equivalent to helix 2 of the ImE7. The alignment of amino acid residues of four E-group immunity proteins is shown in Fig. 1. Sequences of the two overlapping mutagenic oligonucleotide primers for construction of pIE8M are listed as follows:

E29Q D33N E37Q
Forward: 5′-CAAAAAAAACAGAATGATAACCTCCAGC-3′
Reverse: 5′-GGAGGTTATCATTCTGTTTTTTTTGATC-3′

The mismatched nucleotides of the two primers are underlined. The positions of the mutated residues are also shown.

(data not shown) in which the major signatures for "helical conformation" of wild-type and three mutant Im7 proteins (IE7M, IE7-1590 and IE7-2590; Table 1) were 20.25, 19.25, 20.3 and 19.82, respectively. The signatures for "turn conformation" were 6.75, 6.95, 6.87 and 6.8, respectively. The data of CD spectra indicated that conformational change of the mutant Im7 proteins is insignificant. Therefore we predicted that lost of specific inhibitory effect of the mutant Im7 proteins such as IE7M and IE7-1590 are due to the change of individual critical residues for a protein-protein interaction with its cognate colicin and not because of the effect of conformational change in the mutant proteins.

TABLE 2

Phenotype of Site-Directed Mutagenesis of Im7

Plasmid	Mutated residues	Immunity
pUC18	no (no *cei*)	−
pIE7	no (wild-type)	+
pIE7-1	D31N	+
pIE7-2	D32N	+
pIE7-5	D35N	+
pIE7-9	E39Q	+
pIE7-59	D35N+E39Q	+
pIE7-259	D32N+D35N+E39Q	+
pIE7-2590	D32N+D35N+E39Q+D59N	+
pIE7M	D31N+D32N+D35N+E39Q+D59N	−
pIE7-1590	D31N+D35N+E39Q+D59N	−

Note. The substituted residues are shown. The phenotype of the mutant proteins were determined by *in vivo* immunity test (see Materials and Methods) of cells harboring various plasmid. "+" represents cells conferring immunity to colicin E7, while "−" represents cells sensitive to colicin E7.

DISCUSSION

We have demonstrated in this work that three critical residues, D31, D35 and E39 located at loop 1–helix 2 region of the Im7 protein for conferring specific immunity is exhibited in a hierarchical order, respectively. These results suggest that the three residues may play a distinct role in initiating the unique protein-protein interactions with its cognate colicin E7. Existence of the three critical residues for conferring specific immunity is also found in other DNase-type Im proteins.

Recently Wallis *et al.* (17) reported that the five important residues (D30, L33, V34, V37, and E41) from helix 2 of the Im9 are important for the determination of the specific protein–protein interaction. Moreover, we found that mutant protein with multiple mutations in helix 2 of the Im7 still confers specific immunity to colicin E7 (Table 2) unless D31 is included in the multiple combinations of the mutant protein (Table 2). The amino acid sequences alignment showed that residue 31 of Im7 (the last residue of the loop-1) and residues at the corresponding position of three other DNase-type

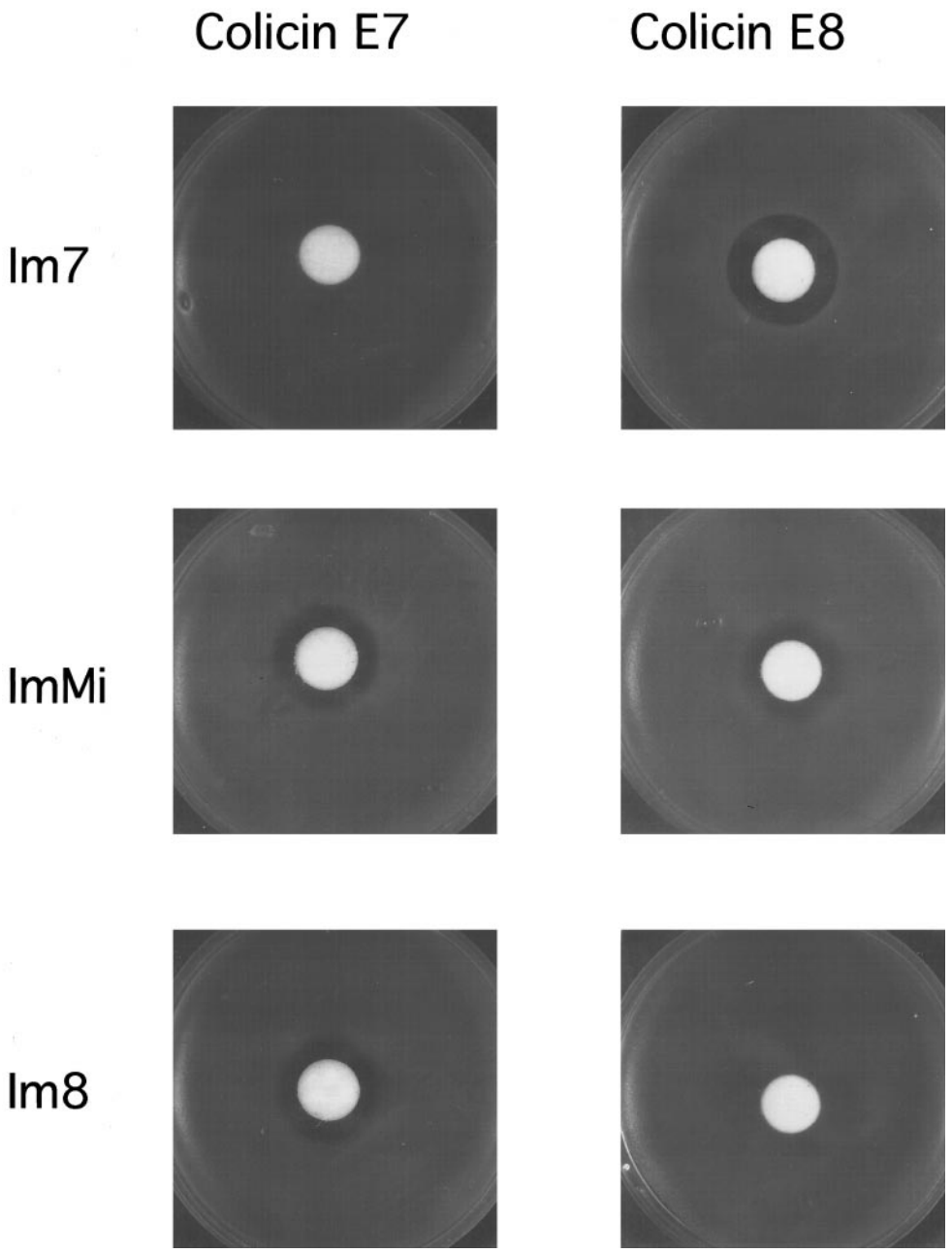

FIG. 4. Identification of helix 2 of Im protein as a dominant specificity–immunity determining region. Helix 2 of Im7 was converted toward Im8 of equivalent by site-directed mutagenesis. The general strategy for construction of ImMi was described in Materials and Methods and the amino acid sequence of the mutant protein is shown in Fig. 1. Three steps of site-directed mutagenesis (see Materials and Methods) achieved the construction of this mutant. The phenotype of the ImMi proteins was determined by *in vivo* immunity test (13). Left panel, immunity assay of cells harboring various plasmids against colicin E7; right panel, immunity assay of cells harboring various plasmids against colicin E8. Im7, cells containing recombinant plasmid with the wild-type ceiE7 gene. ImMi, cells containing recombinant plasmid with the mutant ceiE7 gene in which the nucleotide sequence of the helix 2 region was substituted by the equivalent region of cieE8. Im8, cells containing recombinant plasmid with the wild-type ceiE8 gene. The chimera ImMi protein shows reduced inhibitory activity against ColE7, but increased inhibitory activity against ColE8.

immunity proteins are all negatively charged (Fig. 1). It indicates that the negatively charged residues in this position may play a crucial role both in steric and electrostatic clashes for initiating the unique complex formation. D35 and E39 of the Im7 are the other two least important residues for protein recognition. Both Im7 and Im8 contain negatively charged residues in these two corresponding positions. However, Im2 and Im9 contain hydrophobic, polar or even positively charged residues at the corresponding positions. The

binding affinity study showed that the dissociation constant for the Im9 in complex with its cognate ColE9 DNase is 10^{-14} M, while the non-cognate Im proteins in complex with the ColE9 DNase are 10^{-8} M for Im2, 10^{-6} M for Im8 and 10^{-4} M for Im7(18). Im7 and Im8 bind less tightly with ColE9 which implies that the residues in position D35 and E39 for Im7; D33 and E37 for Im8 must be involved in the stabilization of a protein-protein interaction between an immunity protein and its cognate colicin. Furthermore, these results also indicated that residues on helix 2 as a whole determine the overall immunity specificity (19).

The crystal structure of the DNase-Im7 complex (15) shows that the three critical residues of Im7 interact with the DNase domain by forming six hydrogen bonds in which D31, D35 and E39 make three, two and one hydrogen bonds, respectively. This result has been further confirmed by the recent published crystal structure of the DNase-Im9 complex (20). Thus the results of crystal structure are consistent with the *in vivo* mutagenesis and further confirm that the three critical residues are indeed arranged in a hierarchical order.

In conclusion, all these results suggest that the DNase-type immunity proteins indeed share a homologous structural framework for a protein-protein interaction with their cognate colicins. Thus, evolution of immunity proteins may be engendered just by amino acid changes in this region either by positive selection of diversity or recombinational shuffling without causing any conformational change of the Im proteins. In characterizing the crystal structure of DNase-Im7 complex (15), we found that the three critical residues do not interact directly with the activity site of the DNase domain. How this Im protein inhibits the toxicity of the DNase domain is still remains to be resolved.

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