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Nuclear Magnetic Resonance Structure and Mutational Analysis of the Lactococcin A Immunity Protein

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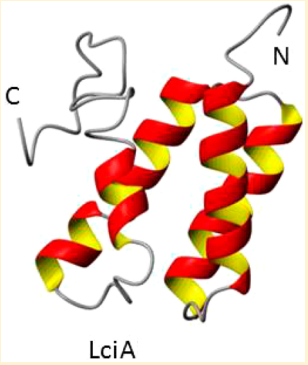
Supporting Information

ABSTRACT: The class IId bacteriocin lactococcin A and the pediocin-like bacteriocins induce membrane leakage and cell death by specifically binding the mannose phophotransferase system (man-PTS) on their target cells. The bacteriocins' cognate immunity proteins that protect the producer cell from its own bacteriocin recognize and bind to the bacteriocin−man-PTS complex and thereby block membrane leakage. In this study, we have determined the three-dimensional structure of the lactococcin A immunity protein (LciA) by the use of nuclear magnetic resonance spectroscopy. LciA forms a four-helix bundle structure with a flexible C-terminal tail. Despite the low degree of sequence similarity between LciA and the pediocin-like immunity proteins, they share the same fold. However, there are certain differences between the structures. The C-terminal helix in LciA is considerably shorter than that observed in the pediocin-like immunity proteins, and the surface potentials of the immunity proteins differ. Truncated variants of LciA in which 6 or 10 of the C-terminal residues were removed yielded a reduced degree of protection, indicating that the unstructured C-terminal tail is important for the functionality of the immunity proteins.



Lactic acid bacteria (LAB) produce a wide variety of antimicrobial peptides, generally termed bacteriocins. Located in the same operon as the bacteriocin gene is a gene encoding an immunity protein that protects the producer from its own bacteriocin.[1] The LAB bacteriocins may be divided into two major classes: class I bacteriocins (also termed lantibiotics) that contain highly modified residues such as lanthionine and class II bacteriocins that do not contain lanthionine.[2] Class II bacteriocins may be further divided into four subclasses: (i) the pediocin-like bacteriocins (class IIa), which have highly similar amino acid sequences and contain a conserved N-terminal YGNGV motif, (ii) the two-peptide bacteriocins (class IIb) that require the complementary action of two different peptides for optimal antimicrobial activity, (iii) the cyclic bacteriocins (class IIc), in which the C- and N-terminal ends are covalently linked, and (iv) the linear, one-peptide bacteriocins (class IId) that share no sequence similarity with the pediocin-like bacteriocins.[2] Many LAB bacteriocins induce cell death by specifically binding to a membrane protein (i.e., the bacteriocin receptor) on the target cell.[3] One of the first receptors to be identified was the mannose phosphotransferase system (man-PTS), which is the receptor for the pediocin-like bacteriocins.[4−6] Upon binding the bacteriocin, the receptor presumably alters its conformation in a manner that leads to membrane leakage and cell death. The immunity protein senses this change in conformation and binds to the bacteriocin−receptor complex and thereby blocks membrane leakage.[4]

The pore-forming class IId bacteriocin lactococcin A,[7] which shows no similarity in sequence to the pediocin-like bacteriocins, also targets the man-PTS.[4] However, lactococcin A displays target cell specificity different from that of the pediocin-like bacteriocins. Lactococcin A seems to target only lactococcal man-PTSs, whereas the pediocin-like bacteriocins recognize the man-PTS of enterococci, lactobacilli, and listeria, but not that of lactococci.[8] Moreover, lactococcin A and the pediocin-like bacteriocins seem to recognize and bind different regions of the receptor. Regions in both the membrane-embedded IIc and IId components of the man-PTS are involved in recognition of lactococcin A, whereas the pediocin-like bacteriocins recognize mainly the IIc component.[9]

The amino acid sequence of the lactococcin A immunity protein (LciA) differs considerably from those of the pediocin-like immunity proteins, yet these immunity proteins have a similar self-protection mechanism (i.e., binding to the bacteriocin−man-PTS complex).[4] The three-dimensional (3D) structures of several immunity proteins that belong to

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the pediocin-like bacteriocins have been determined, and they all fold into an antiparallel four-helix bundle.[10−14] Also, Spy2152, a putative immunity protein produced by *Streptococcus pygenes*, a strain closely related to LAB, folds into an antiparallel four-helix bundle.[15] In this study, we have determined the 3D structure of LciA and investigated the role of the unstructured C-terminal tail by introducing point mutations and creating truncated versions of LciA.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Lactococcus lactis* ssp. *cremoris* LMGT2130,[16] *Lactococcus* LMGT2077, and *Lactococcus lactis* spp. *lactis* IL1403[17] were grown at 30 °C without shaking in M17 medium containing 0.4% glucose (GM17). *Escherichia coli* DH5α and TG1 were grown in LB at 37 °C with vigorous agitation. Ampicillin was added to a final concentration of 100 μg/mL for selection of cells containing plciA-CF. Erythromycin was added to a final concentration of 10 or 150 μg/mL for selection of pMG36e[18] or its derivatives in LAB and *E. coli*, respectively.

Isolation of Genomic and Plasmid DNA. Genomic DNA from *L. lactis* ssp. *cremoris* LMGT2130 was isolated using the DNeasy Tissue kit (Qiagen) according to the protocol for purification of genomic DNA from Gram-positive bacteria. Plasmids were isolated from *E. coli* DH5α or TG1 cells using either the NucleoSpin Plasmid kit or the NucleoBond Xtra Midi kit (both kits from Macherey-Nagel) according to the protocol.

Expression Vector for Cell-Free Protein Synthesis. The gene encoding LciA (*lciA*) was amplified from the genomic DNA of *L. lactis* ssp. *cremoris* LMGT2130, using the primers lciA_pIVEX_F and lciA_pIVEX_R (see Table S1) and the Taq polymerase. The primers contained the restriction sites for *Not*I and *Sac*I, respectively. The polymerase chain reaction (PCR) product was subcloned into the pGEM-T Easy vector (Promega) before it was cloned into the pIVEX2.4d vector (5′PRIME) using the *Not*I and *Sac*I sites, resulting in expression vector plciA-CF where LciA is expressed with an N-terminal His tag and a linker region (MSGSHHHHHHS-SGIEGRGR-LciA). The correct sequence was verified by DNA sequencing, and the structural gene was identical to the *lciA* gene reported by Stoddard et al.[19]

Expression Vector for Activity Measurements. To analyze the activity of the lactococcin A immunity protein, the *lciA* gene was also amplified from genomic DNA using the primers lciA_pMG36e_F and lciA_pMG36e_R (see Table S1) and the Pfu Turbo polymerase. The primers contain the restriction sites for *Sac*I and *Xba*I, respectively. The PCR product and pMG36e were digested with *Sac*I and *Xba*I before ligation. The ligation mixture was used to transform $CaCl_2$-competent *E. coli* TG1 cells. Plasmids were isolated from colonies growing on LB plates containing erythromycin, and the correct sequence was verified by DNA sequencing. The resulting plasmid was termed plciA.

Site-Directed Mutagenesis. Mutations in LciA were introduced using the QuikChange site-directed mutagenesis methodology (Agilent), using plciA as a template. All primers are listed in Table S1. The PCR product was digested with *Dpn*I for 1 h at 37 °C before transformation of competent *E. coli* TG1 cells. Plasmids with the correct mutations in the *lciA* gene were verified by DNA sequencing.

Electroporation of Competent LAB. Competent cells of *Lactococcus* LMGT2077 and *L. lactis* ssp. *lactis* IL1403 were prepared, and electroporation was performed as described by Holo and Nes.[20]

Activity Assay. The degree of resistance conferred by LciA or its mutated variants against lactococcin A was quantified in a microtiter plate assay system as described previously.[21] Each well contained 200 μL of GM17, 2-fold dilutions of lactococcin A, and the indicator strain. Stationary phase cultures of the indicator strains *Lactococcus* LMGT2077 and *L. lactis* IL1403 containing pMG36e, plciA, or the plciA-mutated variants were diluted in a 1:50 ratio before being added to the microtiter plate. The growth inhibition was measured spectrophotometrically (Tecan plate reader) at 600 nm after incubation for 5 h at 30 °C. The plates were then incubated at room temperature, and growth inhibition was measured again after 24 h. The MIC (minimum inhibitory concentration) was defined as the amount of peptide that inhibited growth by 50%.

Cell-Free Protein Synthesis. LciA was produced using a batch mode cell-free system based on an *E. coli* S12 extract as previously described.[22] Screening of expression conditions (with or without detergents present) was conducted in 200 μL reaction volumes in 1.5 mL tubes. The following detergents were tested (final concentrations): 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DH6PC, 0.75%), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DH7PC, 0.2%), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LMPG, 0.01%), and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (LPPG, 0.025%). Large scale cell-free expression reactions (for NMR sample preparation) were performed without detergents present at 30 °C for 2 h at 750 rpm using 50 mL tubes in an Eppendorf Thermomixer. Doubly labeled amino acids (^{13}C and ^{15}N) were purchased from Cambridge Isotope Laboratories. To confirm the production of solubilized LciA, samples of the total and soluble fractions from the cell-free reactions were analyzed by Western blotting. The samples were run on a mini-protean TGX stain-free gel (Bio-Rad), and proteins were transferred onto a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer system from Bio-Rad. The membrane was incubated with THE His tag antibody (Genscript) according to the One Hour Western protocol (Genscript) and developed using LumiSensor (Genscript) chemiluminescent HRP substrate detection in a Fuji LAS-1000plus CCD camera.

Purification of LciA. Soluble LciA from the cell-free reaction was applied on a cOmplete His tag purification column (5 mL, Roche), equilibrated with buffer A [50 mM phosphate buffer and 300 mM NaCl (pH 8)] and connected to a Bio-Rad NGC chromatography system. The column was washed with buffer A, before His-tagged LciA was eluted using a linear gradient of buffer B [buffer A containing 500 mM imidazole (pH 8)]. The eluate was concentrated using Amicon Ultra 4 mL filters (Millipore) with a 3 kDa cutoff and repeatedly spun for 10 min at 4000*g* and 20 °C. Between each spin, the solution was resuspended by pipetting to avoid precipitation. The concentrated protein sample was applied to a Hiload 16/600 Superdex 75 pg column and eluted in 100 mM phosphate buffer containing 100 mM NaCl (pH 7) as a final purification step.

Differential Scanning Fluorimetry (DSF). The thermal stability of LciA in different buffers (MES, HEPES, and phosphate) at variable salt concentrations (50−200 mM NaCl) and pH values (5.5−8) with additives [1 mM DTT, 10% (v/v) glycerol, 15 mM $MgSO_4$, and 5 mM arginine/lysine/glycine] was analyzed by performing a thermal denaturation curve in the

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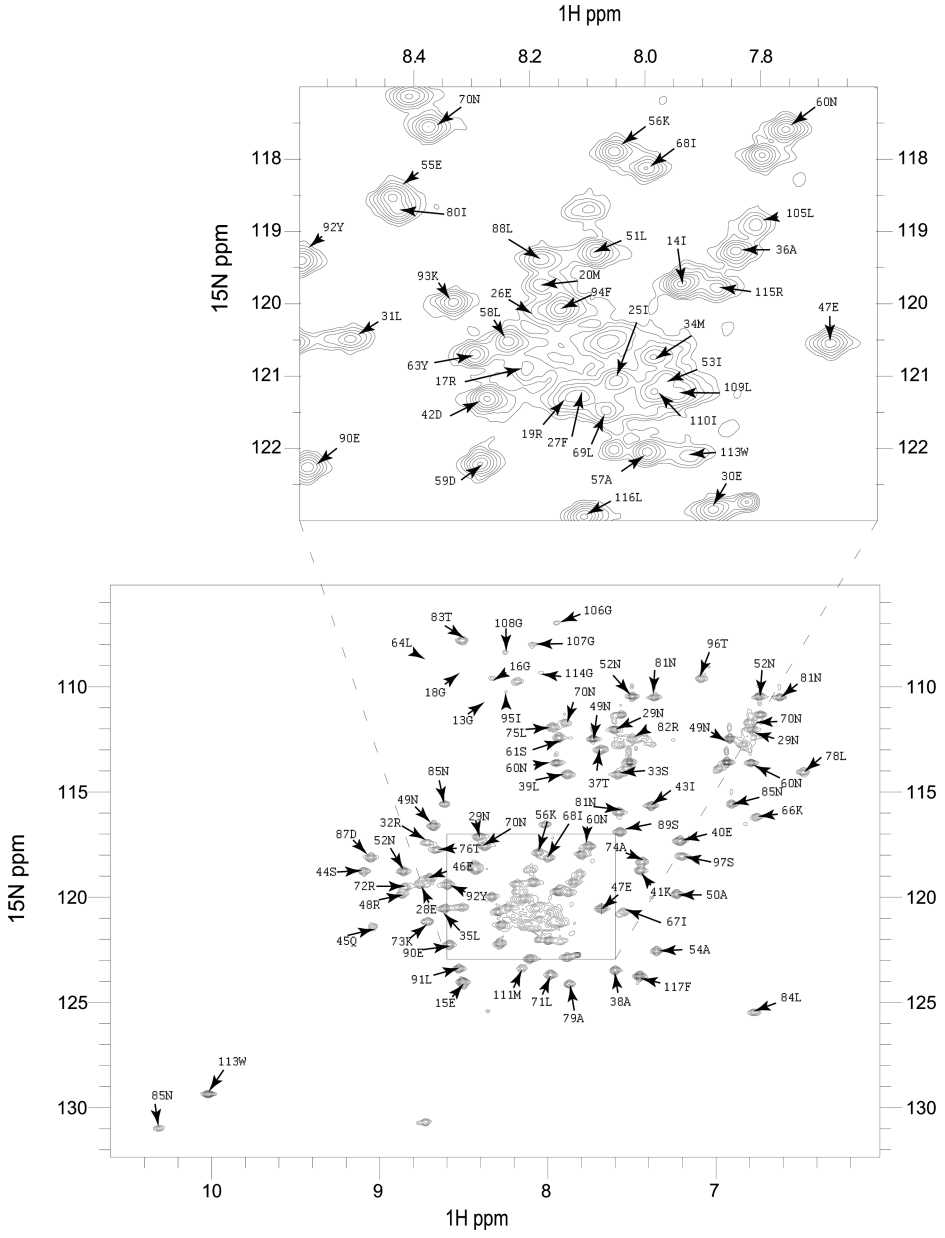

Figure 1. ^{15}N HSQC spectrum of LciA obtained by NMR spectroscopy. Assignment of the full spectrum (bottom) and expansion of the central region (top). Assignment labels are with regard to the full sequence, including the N-terminal tag of 19 amino acids.

presence of SYPRO Orange (90× concentration, from a 5000× stock in DMSO, Life Technologies). The temperature ranged from 4 to 90 °C.

NMR Measurements. Samples of LciA (~0.6 mM) were prepared in a 90% H_2O/10% D_2O solvent or 100% D_2O with 100 mM sodium phosphate (pH 7.0) and 100 mM NaCl. Sequential assignment was obtained using the standard set of backbone experiments (HNCO, HNCA, HNcoCA, HNCACB, and HNcoCACB[23]) performed on an 800 MHz Bruker Avance HDIII NMR spectrometer with a TXO cold probe. Side chain assignments were obtained from (H)CCH tocsy. Structure information was obtained from three-dimensional (3D) ^{15}N NOESY-HSQC and 3D ^{13}C NOESY-HSQC experiments. All NMR experiments were performed at 25 °C, and NMR data were analyzed using the CCPN package.[24]

Titration of LciA with DH7PC. DH7PC was dissolved in nuclease-free water and added to the LciA NMR sample. ^{15}N HSQC spectra of LciA were recorded in the presence of 0.2% DH7PC.

Structure Calculations. Structures of LciA were calculated using CYANA version 3.97.[25] NOE distance restraints combined with dihedral restraints and hydrogen bond distance constraints in the α-helices derived from TALOS[26] were used to calculate ensemble model structures. A total of 100 structures were calculated, and after water refinement (conducted in CNS 1.3 with waterRefCNS scripts according to the NESG protocol[27]), the 16 lowest-energy structures were selected and analyzed. Molecular graphics were produced using MOLMOL[28] and PyMol.[29] The electrostatic surfaces were calculated using PDB2PQR[31] and APBS.[30−32] The NMR-derived ensemble structures were deposited as PDB entry 5LFI.

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Figure 2. Sequence plot of short distance and torsion angle restraints obtained from the NOESY spectrum of LciA and TALOS analysis of the chemical shifts of LciA, respectively. Only unique distance restraints are shown. Cross-peaks that show correlation between two protons but are too small to contain structural information are not shown. The line thicknesses are related to NOE cross-peak intensities. ϕ and ψ angles obtained from TALOS are indicated with asterisks and circles. An asterisk indicates that the torsion angle restraint is in agreement with the values expected for the α-helical region of the Ramachandran plot.

RESULTS AND DISCUSSION

Production and Stability of LciA. On the basis of its amino acid sequence, LciA is most likely a cytosolic protein. However, previous studies have suggested that it might partly associate with the cell membrane[33] or possibly contain one transmembrane helix.[34] Addition of various detergents to the mixtures for the cell-free protein synthesis reactions did not increase the amount of solubilized LciA compared to that seen for the reaction without detergents, as judged by Western blotting of the total and soluble fractions from the cell-free reactions (results not shown). Various buffers (HEPES, MES, and phosphate), NaCl concentrations (50−200 mM), pH values (5.5−8), and additives [1 mM DTT, 10% (v/v) glycerol, 15 mM $MgSO_4$, and 5 mM arginine/lysine/glycine] were tested to determine the optimal conditions for thermally stable LciA. Neither of these parameters had a large effect on the T_m of LciA [the T_m varied slightly between 58 and 62 °C (see Table S2)]. The T_m value using a 100 mM phosphate buffer with 100 mM NaCl (pH 7) was slightly higher than for the other conditions. This buffer was consequently chosen as the NMR sample buffer. For NMR sample preparation, LciA was produced without detergents or other additives present. To test if LciA in fact contains a membrane helix, $^1H-^{15}N$ HSQC experiments were conducted in water and in the presence of 0.2% DH7PC. We observed no change in the chemical shifts as a function of the addition of membrane mimics (results not shown), indicating that the protein does not contain transmembrane helices.

LciA Forms a Four-Helix Bundle. The assigned ^{15}N HSQC spectrum of LciA with the N-terminal His tag and linker sequence is shown in Figure 1. A sequence plot of short distance restraints obtained from NOESY and torsion angle restraints obtained from TALOS is shown in Figure 2. The His tag and linker region are not included in Figure 2, and the numbering of amino acids in this figure and in the remaining text corresponds to that of wild-type LciA. The sequence plot shows that there are possible helices from Q5 to E21, Q26 to S42, K47 to A55, and S70 to T77. The TALOS torsion angles are mostly in the α-helical region of the Ramachandran plot, indicating torsion angles in agreement with an α-helix in the same regions as the sequence plot. Distance restraints from NOESY and torsion angle restraints from TALOS were used to calculate the 3D structure of LciA using CYANA before the structures were refined in water. A total of 16 structures after water refinement were deposited as PDB entry 5LFI, while the structural restraints and chemical shifts were deposited as Biomagnetic Resonance Data Bank entry 34018. The number and type of structural restraints and the structural statistics obtained for LciA are listed in Table 1.

The N-terminal His tag and linker sequence of the protein were found to be unstructured, in agreement with the results of $^1H-^{15}N$ NOE experiments and TALOS analysis of the chemical shifts. The structural ensemble is shown in Figure

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Table 1. Numbers and Types of Structural Restraints and Structural Statistics Obtained for LciA

no. of constraints	
total no. of NOE constraints	1351
short-range	63
medium-range ($1 < \|i - j\| < 5$)	305
long-range ($\|i - j\| > 5$)	283
no. of dihedral angle constraints	136
ϕ	68
ψ	68
total no. of constraints	1487
structural statistics	
constraint violations	
distance constraints (Å)	0
dihedral angle constraints (deg)	0
RMSD from mean structure of residues 5−75	
backbone (Å)	0.73 ± 0.15
heavy atoms (Å)	1.49 ± 0.12
Ramachandran statistics (%)	
core regions	81.2
allowed regions	18.7
generous regions	0
disallowed regions	0
CYANA statistics	
no. of peaks used in calculation	2463
no. of assigned peaks	2351
no. of unassigned peaks	112
no. of manually assigned peaks	0
target function	1.07 ± 0.026

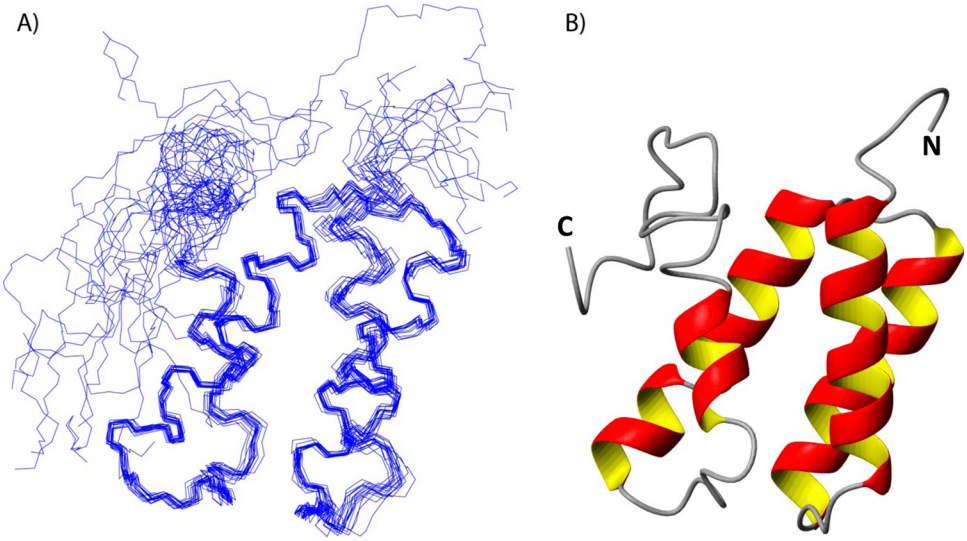

Figure 3. 3D structure of LciA. The N-terminal His tag and linker sequence are not included. (A) Structural ensemble. (B) Cartoon drawing of the lowest-energy structure in the structural ensemble.

3A, while the cartoon depiction of the lowest-energy structure is shown in Figure 3B. LciA forms an antiparallel four-helix bundle (Figure 3), with a flexible C-terminal tail. The α-helices are α1 between residues E7 and E21, α2 between residues Q26 and N41, α3 between residues L44 and N62, and α4 between residues S70 and F75. The lack of structure in the C-terminal tail of LciA is in part confirmed by the higher mobility in the $^{1}H-^{15}N$ NOESY experiments and chemical shift analysis by TALOS.

LciA Stucture Compared to Those of Other Immunity Proteins with a Similar Fold. The four-helix bundle fold found for LciA is similar to the fold observed for the immunity proteins of the pediocin-like bacteriocins.[10−14] Interestingly, LciA and the pediocin-like immunity proteins function in a similar manner. They bind to the bacteriocin−man-PTS complex and prevent membrane leakage.[4] The molecular structure and surface potential of LciA were compared to those of a pediocin-like immunity protein (ImB2, 12.5% identical to LciA) and a putative immunity protein Spy2152 (16% identical to LciA) that also form a four-helix bundle structure[15] to investigate the extent of similarity between these immunity proteins (Figure 4). The RMSDs between the

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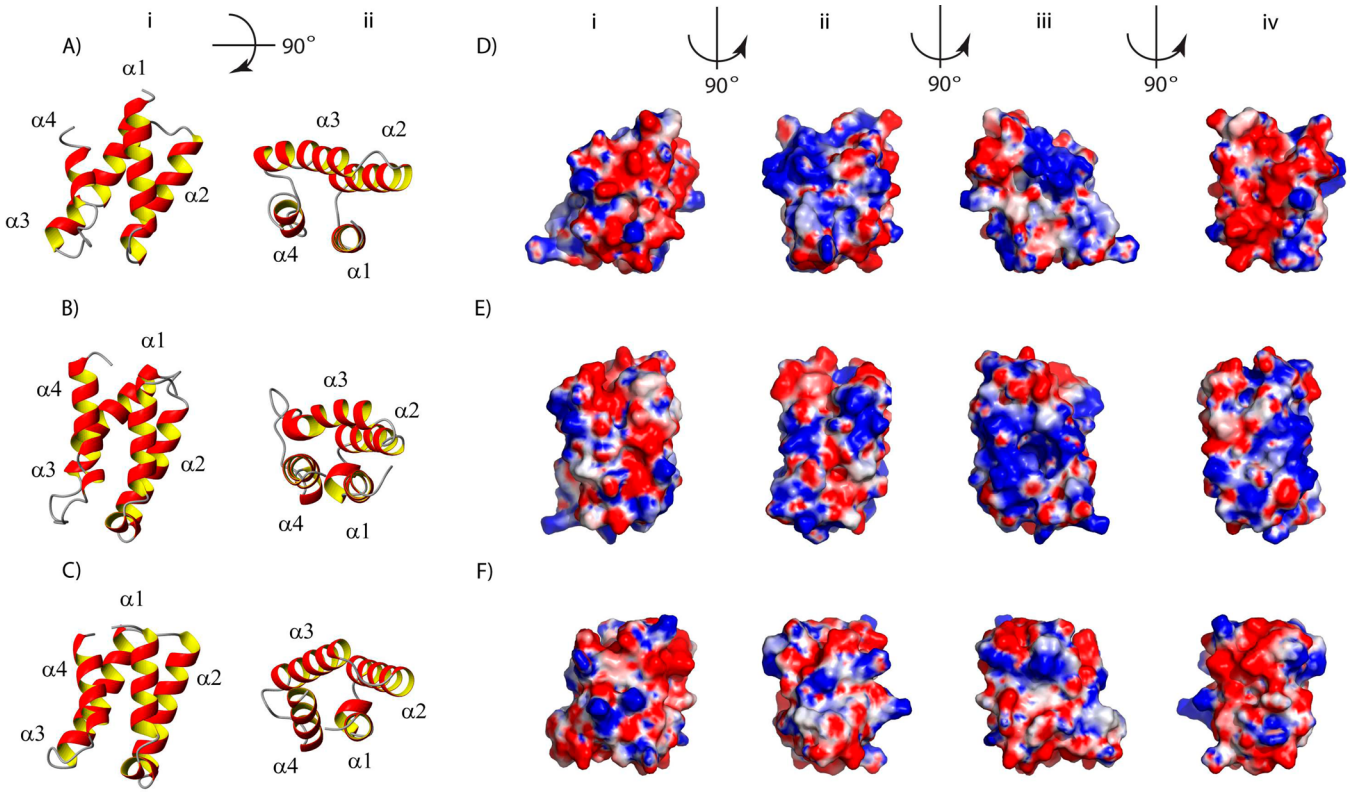

Figure 4. Structural comparison of three immunity proteins. Cartoon pictures of the structured four-helix domains of (A) the lactococcin A immunity protein, (B) the carnobacteriocin B2 immunity protein, and (C) the putative immunity protein Spy2152. The four α-helices are labeled α1−α4. The cartoon pictures are shown with helices α4 and α1 in front (i) and from the top (ii). Electrostatic surfaces of (D) the lactococcin A immunity protein, (E) the carnobacteriocin B2 immunity protein, and (F) the putative immunity protein Spy2152. The orientations of the surface structures are the same in each column. In column i the surfaces of α4 and α1 are shown, in column ii the surfaces of α3 and α4, in column iii the surfaces of α2 and α3, and in column iv the surfaces of α1 and α2. Positively charged electrostatic surface areas are colored blue, negatively charged electrostatic surface areas red, and noncharged areas of the surface gray. The arrows and numbers illustrate the axes of rotation and the direction and angle of rotation between the two depictions.

structural domains of LciA and ImB2, LciA and Spy2152, and ImB2 and Spy2152 were 2.9, 3.7, and 2.7, respectively. Although the three immunity proteins share the four-helix bundle structure, the lengths and orientations of the helices are slightly different (Figure 4). While LciA helices α1−α4 are 15, 16, 18, and 6 residues long, respectively, the helices in ImB2 and Spy2152 are 13, 15, 17, and 12 and 12, 17, 17, and 12 residues long, respectively. The C-terminal helix of LciA is thus shorter than the helices in the two other immunity proteins. All immunity proteins that have been structurally characterized have C-terminal tails that are of random structure. Furthermore, ImB2, in contrast to the other immunity proteins, has a fifth C-terminal nine-residue α-helix. Neither the fifth helix nor the C-terminal unstructured tails of the proteins are shown in Figure 4. The relative angles between helices α1 and α2 are 150°, 163°, and 155° for LciA, ImB2, and Spy2152, respectively. Similarly, the angles for the other helices are 167°, 153°, and 162° between α2 and α3, 124°, 128°, and 136° between α3 and α4, and 157°, 170°, and 147° between α4 and α1, respectively.

While the overall fold of the three proteins shows certain similarities, the surface potentials are different (Figure 4D−F). In LciA, there are electropositive and electronegative areas somewhat scattered around on the surface (Figure 4D), with a relatively large electronegative area at the end of α1 and α4 consisting of residues E7, E9, E11, and E71 (Figure 4D, i) and a smaller electropositive patch at the start of α3 consisting of residues K47, R53, and K54 (Figure 4D, iii). In ImB2, there are an overall mostly electropositive side and an electronegative side to the protein as shown in Figure 4E. Furthermore, an extended electronegative patch is observed all along the side of the protein on and between α1 and α4 (Figure 4E, i), and a similar electropositive patch is observed at the start of α2 and the end of α3 (Figure 4E, iii). Spy2152 has a less well-defined electronegative patch between α1 and α4 (Figure 4F, i) and a smaller electropositive patch at the start of α3 (Figure 4F, iii). Except for the fact that there is a more electronegative patch on the immunity proteins between helices 1 and 4 and to some degree an electropositive area at the start of helix 3, there are no obvious similarities in electrostatic potential among the three immunity proteins shown in Figure 4D−F.

The Flexible C-Terminal End is Important for the Functionality of LciA. By generating hybrid immunity proteins in which the C- and N-terminal parts were derived from different immunity proteins, it has been shown that the C-terminal halves of the immunity proteins of the pediocin-like bacteriocins are responsible for recognition of the cognate bacteriocin and receptor complex.[35,36] Furthermore, mutational studies of Mun-im[10] and PedB[12] have indicated that the unstructured C-terminal ends of these pediocin-like immunity proteins are important for immunity. To investigate the role of the unstructured C-terminal tail of LciA, point mutations were introduced at the C-terminal end and three truncated variants of LciA were constructed (deletion of 2, 6, or 10 residues from

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Table 2. MIC Values and Degrees of Resistance Conferred by LciA and Its Mutant Versions

	Lactococcus LMGT2077				*L. lactis* ssp. *lactis* IL1403			
immunity protein	MIC (nM)[a] value (5 h)	degree of resistance[b] (5 h)	MIC (nM)[a] value (24 h)	degree of resistance[b] (24 h)	MIC (nM)[a] value (5 h)	degree of resistance[b] (5 h)	MIC (nM)[a] value (24 h)	Degree of resistance[b] (24 h)
–[c]	0.07 ± 0.04	1	0.3 ± 0.2	1	0.04 ± 0.02	1	0.10 ± 0.05	1
LciA	74 ± 25	1100	150 ± 55	500	10 ± 5	250	45 ± 10	450
LciA(Δ2C)[d]	60 ± 30	860	140 ± 50	470	15 ± 9	380	31 ± 11	310
LciA(Δ6C)	45 ± 26	640	37 ± 13	120	8 ± 4	200	7 ± 4	70
LciA(Δ10C)	0.5 ± 0.3	7	0.9 ± 0.4	3	0.4 ± 0.3	10	1.1 ± 0.3	11
LciA(K2A)	93 ± 41	1300	160 ± 60	530	24 ± 9	600	80 ± 12	800
LciA(W94A)	140 ± 50	2000	230 ± 60	770	11 ± 6	280	37 ± 9	370
LciA(R96A)	80 ± 40	1100	165 ± 53	550	7 ± 1	180	25 ± 5	250

[a]The MIC values are given as the average of three or four independent measurements ± the standard deviation. Growth inhibition was measured after 5 or 24 h. [b]The degree of resistance is defined as the MIC value obtained when the recombinant strain contained the plciA or a mutated version of the plasmid divided by the MIC value obtained for the strain harboring the unmodified pMG36e plasmid. [c]No immunity protein; the strain contains the empty pMG36e plasmid. [d]Δ*n*C symbolizes the deletion of *n* amino acids from the C-terminal end.

the C-terminal end). Two lactococcin A-sensitive strains of lactococci were transformed with pMG36e, plciA, or mutated versions of plciA and assayed against lactococcin A to test the degree of protection conferred by the lactococcin A immunity protein or its mutated variants. The degree of protection was evaluated by comparing the MIC values obtained when the strain expressed the (mutated) immunity protein with the MIC values obtained when the same strain contained the unmodified pMG36e plasmid. When the strains expressed the lactococcin A immunity protein, a 250−1100 fold increase [depending on the strain (Table 2)] in the concentration of lactococcin A was required to obtain an antimicrobial activity similar to that obtained with the strains not expressing the immunity protein. Replacing Trp94 or a positively charged residue at the N- or C-terminal end (Lys2 or Arg96, respectively) with Ala did not have a marked effect on the degree of protection conferred by the lactococcin A immunity protein, nor did the removal of the two C-terminal residues (Table 2). However, removing either 6 or 10 residues from the unstructured C-terminal end affected the functionality of the immunity protein. Compared to that of the wild-type immunity protein, the degree of resistance conferred by the immunity protein in which six of the C-terminal residues were removed was somewhat reduced after 5 h. After incubation for 24 h, the degree of protection was further reduced by a factor of 4−6 [depending on the strain (Table 2)]. Removing the 10 C-terminal residues from the immunity protein was even more detrimental, resulting in an only 3−11-fold degree of protection (depending on the strain) after both 5 and 24 h (Table 2).

Although they have only a low degree of sequence similarity, the immunity proteins that prevent cell death by binding the bacteriocin−man-PTS complex and whose structure has been determined all form a four-helix bundle structure with an unstructured C-terminal tail. This C-terminal tail seems to be important for the functionality of the immunity proteins. Also, LciB, the immunity protein of another class IId bacteriocin (lactococcin B), confers immunity by binding to the man-PTS system.[4] On the basis of sequence similarities between LciB and LciA (approximately 46%), it is likely that also LciB folds into a four-helix bundle structure.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00848.

A list of all primers used in this study and a table of the T_m values of LciA in various buffers and under various conditions (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DSF, differential scanning fluorimetry; DH7PC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; LAB, lactic acid bacteria; LciA, lactococcin A immunity protein; man-PTS, mannose phosphotransferase system; MIC, minimum inhibitory concentration; NMR, nuclear magnetic resonance; RMSD, root-mean-square deviation.

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