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Much of the Microcin J25 Leader Peptide is Dispensable

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Microcin J25 (MccJ25) is a 21 amino acid (aa) antimicrobial peptide[1,2] targeting RNA polymerase[3−5] that exhibits a remarkable "lasso" topology in which the N-terminus of the peptide is covalently linked to a glutamic acid side chain (Figure 1A).[6−8] The in vitro conversion of the linear 58 aa MccJ25 precursor, McjA, into the matured lasso form requires two enzymes, McjB and McjC (Figure 1B).[9,10] Production of MccJ25 in its native expression host, *Escherichia coli*, requires an additional protein, McjD, that serves as an immunity factor by putatively pumping the mature antibiotic out of the cytoplasm.[11,12] While the precise biochemical functions of McjB and McjC have not yet been elucidated, bioinformatics studies[9] indicate that McjB is the putative protease that cleaves McjA. Formation of the MccJ25 isopeptide bond is hypothesized to be catalyzed by McjC, which shares some homology with several amide-forming enzymes, including asparagine synthetases. The role of the leader sequence of McjA (Figure 1D), which spans 37 aa including the N-terminal methionine, has also remained obscure. In this paper, we demonstrate that much of this leader sequence is dispensable; the minimal leader peptide for maturation of McjA into MccJ25 is composed of only the eight residues at the C-terminus of the leader peptide.

To evaluate the effects of truncations of the McjA leader peptide, we utilized an engineered gene cluster containing the four genes required for MccJ25 biosynthesis (Figure 1C). In this gene cluster, which is harbored on the plasmid pJP3, *mcjA* expression is driven by an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible T5 promoter, and the *mcjBCD* operon is under the control of its natural promoter.[13] A collection of truncation variants in which 5 aa segments were removed from the N-terminus of the leader peptide (Figure 1D) was cloned into pJP3, and the resulting plasmids were transformed into the *E. coli* expression host DH5α. Transformants were grown to midexponential phase in LB medium, induced with 1 mM IPTG, and allowed to produce MccJ25 overnight (20 h) at 37 °C. To assess the level of MccJ25 produced by each of the truncation variants, a spot-on-lawn assay was performed essentially as described elsewhere.[9] Briefly, the culture supernatant was obtained via centrifugation and boiled to ensure that MccJ25 was the only bactericidal component in the supernatant. These supernatants were spotted on an agar plate overlaid with soft agar impregnated with exponentially growing *Salmonella newport* (*Salmonella enterica* subsp. *enterica* serovar Newport), a strain of bacteria hypersensitive to MccJ25.[1] Finally, the plates were incubated overnight at 37 °C. In this assay, the size of the zone of *S. newport* growth inhibition qualitatively reflects the level of MccJ25 production.

McjA variants with 5 or 10 aa removed from their N-termini [McjA(6−57) and McjA(11−57), respectively; numbering excludes the N-terminal methionine] exhibit a zone of inhibition essentially the same size as that of full-length McjA (data not shown). Further truncation of 15 aa [McjA(16−57)] or 20 aa [McjA(21−57)] from the N-terminus of McjA led to zones of inhibition slightly smaller

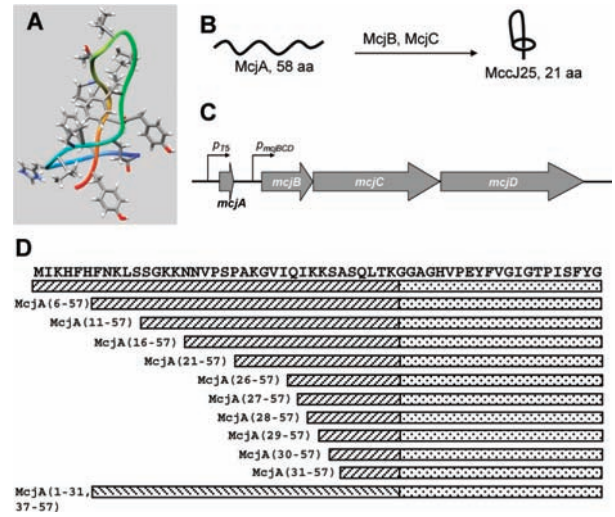

Figure 1. (A) Lasso structure of microcin J25 from PDB file 1Q71. (B) Linear 58 aa McjA preprotein is matured into 21 aa MccJ25 by McjB and McjC enzymes. (C) Schematic of the artificial MccJ25 gene cluster used to produce and mature wild-type McjA and McjA truncations. The *mcjA* gene is placed under the control of an IPTG-inducible T5 promoter, and the *mcjBCD* operon is under the control of its natural promoter. (D) Sequence of McjA and schematic of the truncation variants discussed in this study.

than that observed for full-length McjA (Figure 2). The McjA(26−57) construct, representing a truncation of 25 aa from the N-terminus of full-length McjA, still produced MccJ25 as judged by the spot-on-lawn assay, but the McjA(31−57) construct did not exhibit any MccJ25 production. To determine the minimal leader peptide length for McjA maturation, we further truncated the McjA(26−57) construct one amino acid at a time. These variants displayed a monotonic decrease in the size of the zone of inhibition, with the last functional truncation being the McjA(29−57) variant (Figure 2). These results demonstrate that remarkably, only eight of the 36 residues in the McjA leader peptide are required for processing of the protein by McjB and McjC into mature MccJ25.

To quantify the decrease in MccJ25 productivity due to truncation of the leader peptide, we measured the MccJ25 level using two different methods. First, culture supernatants containing MccJ25 were extracted with *n*-butanol. This organic extract was concentrated under reduced pressure and subjected to HPLC analysis. Since MccJ25 gives a peak that is well-isolated from other peaks, the area under the peak can be used as a measure of the MccJ25 level in the extract. We compared the level of MccJ25 produced from full-length McjA to that produced from McjA(16−57) and McjA(26−57) and found that these truncations led to ∼5- and ∼13-fold reductions, respectively, in the production of MccJ25 (Table 1). The product of the McjA(26−57) construct was analyzed by mass spectrometry, which confirmed that it is authentic MccJ25 (Figure S1 in the Supporting Information). As an alternative

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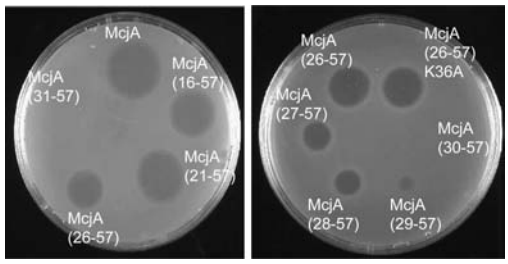

Figure 2. *S. newport* zone of growth inhibition assays for McjA truncation variants: (left) zones of inhibition for full-length McjA and N-terminal truncations of 15, 20, 25, and 30 aa; (right) zones of inhibition for McjA N-terminal truncations of 25−29 aa. The minimal peptide for maturation of MccJ25 is McjA(29−57). The zone of inhibition for the McjA(26−57) K36A construct is also shown in the right panel.

measure of the production level of MccJ25, the culture supernatants from DH5α harboring full-length McjA, McjA(16−57), and McjA(26−57) were serially diluted and spotted on *S. newport* lawns as described above. The last dilution that resulted in a spot was compared and taken as a relative measure of the MccJ25 production level (Table 1 and Figure S2). This analysis indicated that the 15 aa and 25 aa truncations of McjA resulted in 4- and 16-fold reductions in productivity, respectively. Similar dilution analysis of the culture supernatant of cells harboring McjA(29−57), which contains the absolute minimal leader peptide for MccJ25 maturation, indicated that the yield of MccJ25 produced from this truncation variant was ∼256-fold lower than that of full-length McjA.

Table 1. Fold Decrease in MccJ25 Production Level Due to McjA Truncation via HPLC and Spot Assay

	fold decrease in MccJ25 production level	
construct	via HPLC	via spot assay
McjA	1	1
McjA(16−57)	4.9	4
McjA(26−57)	13.2	16
McjA(29−57)	n.d.[a]	256

[a] n.d.: not determined.

One striking aspect of the McjA leader peptide is its high content of basic amino acids; the peptide contains eight lysine residues within the 37 aa of the leader peptide (Figure 1D). To investigate the potential role of these positively charged moieties in MccJ25 maturation, we constructed single lysine-to-alanine point mutants in the McjA(26−57) truncation variant. In addition to constructing the three possible single mutants K28A, K29A, and K36A, we also made a K28A K29A double mutant. Surprisingly, expression of each of these variants gave a spot equivalent in size to that from wild-type McjA(26−57) (Figure 2 and Figure S3), indicating that the lysines in the C-terminal region of the McjA leader sequence are dispensable in regard to the maturation of MccJ25. As an additional control, we constructed an McjA variant in which the five C-terminal residues of the McjA leader sequence were truncated, resulting in the McjA(1−31,37−57) variant (Figure 1D). Cells harboring this variant produced no detectable MccJ25. To further investigate the role of the C-terminal portion of the leader sequence, we constructed the rest of the possible alanine variants in the McjA(26−57) construct. While the S30A and Q33A amino acid changes did not affect MccJ25 production, the S32A and L34A changes led to lower production titers of MccJ25, and the T35A variant did not produce any MccJ25 (Figure S4). Collectively, these results indicate that a determinant for MccJ25 maturation resides within the last ∼10 residues of the McjA leader peptide. Electrostatic interactions between McjA and the maturation proteins McjB and McjC likely do not play a prominent role in this determinant, but hydrogen bonding to the Ser-32 and Thr-35 side chains may play a role in the docking of McjA into the maturation enzymes.

In the absence of detailed structural information about the MccJ25 maturation enzymes McjB and McjC, the results herein provide some insight into the likely role of the McjA leader peptide. The requirement for a minimum of eight residues immediately preceding the cleavage site of McjA indicates that these residues are important for recognition of McjA by the maturation machinery. Thus, one role for the leader peptide is to assist in the docking of McjA in one (or both) of the maturation enzymes. A second possible role of the leader peptide is as a chaperone that assists in the formation of the MccJ25 lasso structure. Our finding that 28 of the 37 residues in the McjA leader peptide can be deleted without abolishment of MccJ25 maturation points to the conclusion that the leader peptide likely does not perform a chaperone function, though this possibility cannot be ruled out completely. Our observations in this paper recall previous work demonstrating that only the C-terminal portion of the leader peptide of the lantibiotic lacticin 481 is required for its maturation.[14] As additional lasso peptides[15] and gene clusters that encode their maturation[16] are discovered, the work presented herein promises to shed light on the posttranslational processing of this fascinating class of peptides.

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Supporting Information Available: Detailed materials and methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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