# Maturation of McjA precursor peptide into active microcin MccJ25†

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**Microcin J25 is a ribosomally synthesised 21-residue antimicrobial peptide produced by certain strains of enterobacteria, that adopts an extraordinary '*threaded lasso*' structure. To date, the biosynthesis of this peptide is little understood. Here we report the *in vitro* maturation of the microcin precursor peptide into active microcin J25 for the first time. Furthermore, we show that the enzymes required for the posttranslational modification of this precursor peptide are associated with the bacterial inner membrane.**

Microcins are a family of antibacterial peptides produced by enterobacteria such as *Escherichia coli* which display potent activity against Gram-negative bacteria related to the producer strain.[1] They are synthesised on the ribosome as precursors, and undergo further enzymatic posttranslational modification which generates a family displaying a wide range of unusual structural features.

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† Electronic supplementary information (ESI) available: Cloning, expression and purification of KSI–McjA fusion protein, chemical cleavage of the KSI-McjA fusion protein and purification of McjA, Western blot analysis demonstrating McjC is membrane associated and purification of MccJ25. See DOI: 10.1039/b708478a

Microcin J25 (MccJ25) is a gene-encoded, hydrophobic 21 amino acid peptide, originally isolated from the culture medium of *Escherichia coli* strain AY25 that adopts an extraordinary three dimensional lariat structure, termed '*a threaded lasso*' (Fig. 1).[2] The cyclic peptide contains an amide bond between the N-terminal amino group and the γ-carbonyl group of Glu8 and, as a result, the first 8 amino acids form a closed ring.[3–5] The C-terminal 12 amino acids of the peptide contain a tight β-hairpin and the C-terminal tail is threaded back through

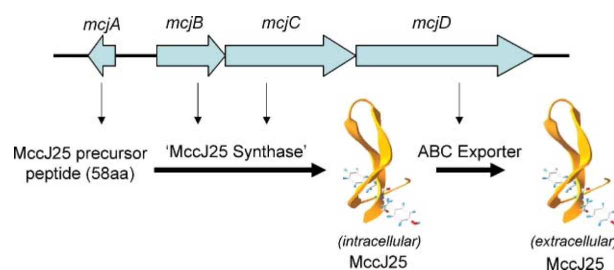


**Fig. 1** The microcin J25 biosynthetic operon. The proposed role of each gene product is highlighted. *McjA* encodes a 58 amino acid precursor peptide. *McjB* and *mcjC* are involved in posttranslational modification of the McjA protein to produce MccJ25, and together are termed "MccJ25 synthase". McjD encodes a proposed ABC transporter which is believed to be responsible for export of mature MccJ25. The MccJ25 NMR structure (PDB code: 1Q71) is adapted from reference 4.

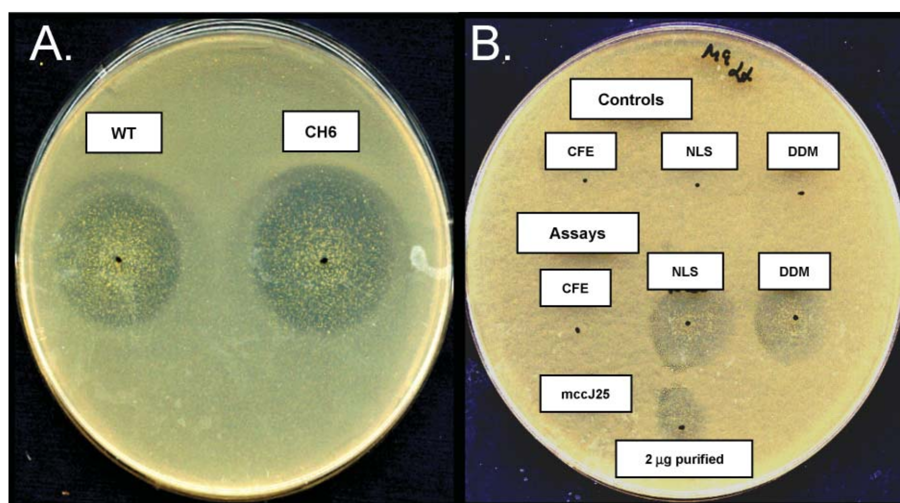


**Fig. 2** (A) Bioassay for MccJ25 production. Single colonies of *E. coli* Novablue transformed with plasmids—wild type *pTUC202* (WT, left) or *pTUC203C6H* (CH6, right) were stabbed into minimal agar and pre-grown for 16 hours at 37 °C before they were overlaid with top agar containing 1 × $10^6$ colony forming units (CFUs) of *E. coli* DH5α sensitive cells. (B) Bioassay for MccJ25 synthase activity. Top row; negative controls of cell free extract (CFE), NLS and DDM. Middle row, assays; CFE, NLS and DDM membrane extraction of pTUC203C6H expressing cells were assayed for their ability to mature McjA into active MccJ25. McjA treated with either NLS and DDM extracted membranes displayed antimicrobial activity whereas the soluble CFE was inactive. Bottom row, positive control, 2 μg purified MccJ25 isolated by reverse-phase chromatography was active.

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the ring. The aromatic residues Phe19 and Tyr20 flank each side of the ring, sterically preventing slippage of the lasso. This remarkable structure is exceptionally stable—MccJ25 is resistant to strong denaturing conditions, proteolysis, and temperatures in excess of 100 °C.[6] MccJ25 enters the cell *via* protein receptors (*e.g.* FhuA) and the primary target is DNA-dependant RNA polymerase (RNAP). The peptide binds to the secondary channel of RNAP, inhibiting access of substrates to the RNAP complex.[7,8]

Four genes, *mcjABCD*, encoded on a large, low-copy number plasmid, are required to produce and export MccJ25 in its active form (Fig. 1).[9] The *mcjA* gene encodes a 58 amino acid MccJ25 precursor peptide; the first 37 residues form a leader peptide which is cleaved during maturation (see supplementary information†).[10,11] *McjD* encodes a protein with 6 predicted transmembrane regions and a C-terminal ATP binding domain, characteristic of bacterial ABC transporters.[10] Biochemical studies have shown that *McjD* expression is not necessary for the biosynthesis of MccJ25, but is required for secretion of the active antibiotic peptide out of the producing cell. Both the gene products of *mcjB* and *mcjC* are required for MccJ25 maturation, which implies they are responsible for cleavage of the leader peptide and cyclisation of the ring, in effect the breaking and making of two amide bonds. To date very little is known about these enzymes, or how they form the machinery responsible for the biosynthesis of MccJ25 (an "MccJ25 synthase"). Here we present, for the first time, the *in vitro* reconstitution of MccJ25 synthase activity. By using a membrane protein extract from *E. coli* cells expressing the *mcjABCD* operon we have successfully matured a recombinant McjA precursor peptide into active MccJ25.

To be able to monitor expression of the operon, site directed mutagenesis was used to introduce a hexahistidine tag at the N-terminus of the *mcjC* gene within the MccJ25 operon. This operon was cloned into a pACY derived plasmid, named *pTUC203C6H*. Cells (*E. coli* DH5α) transformed with this plasmid produced MccJ25 in comparable amounts to the wild type plasmid as judged by a spot-on-lawn antimicrobial assay using a susceptible strain of *E. coli* (Fig. 2A). These cells were lysed by sonication and the supernatant prepared by centrifugation. The resultant lysate was further subjected to ultracentrifugation at 100 000*g* for two hours at 4 °C and soluble supernatant was decanted.

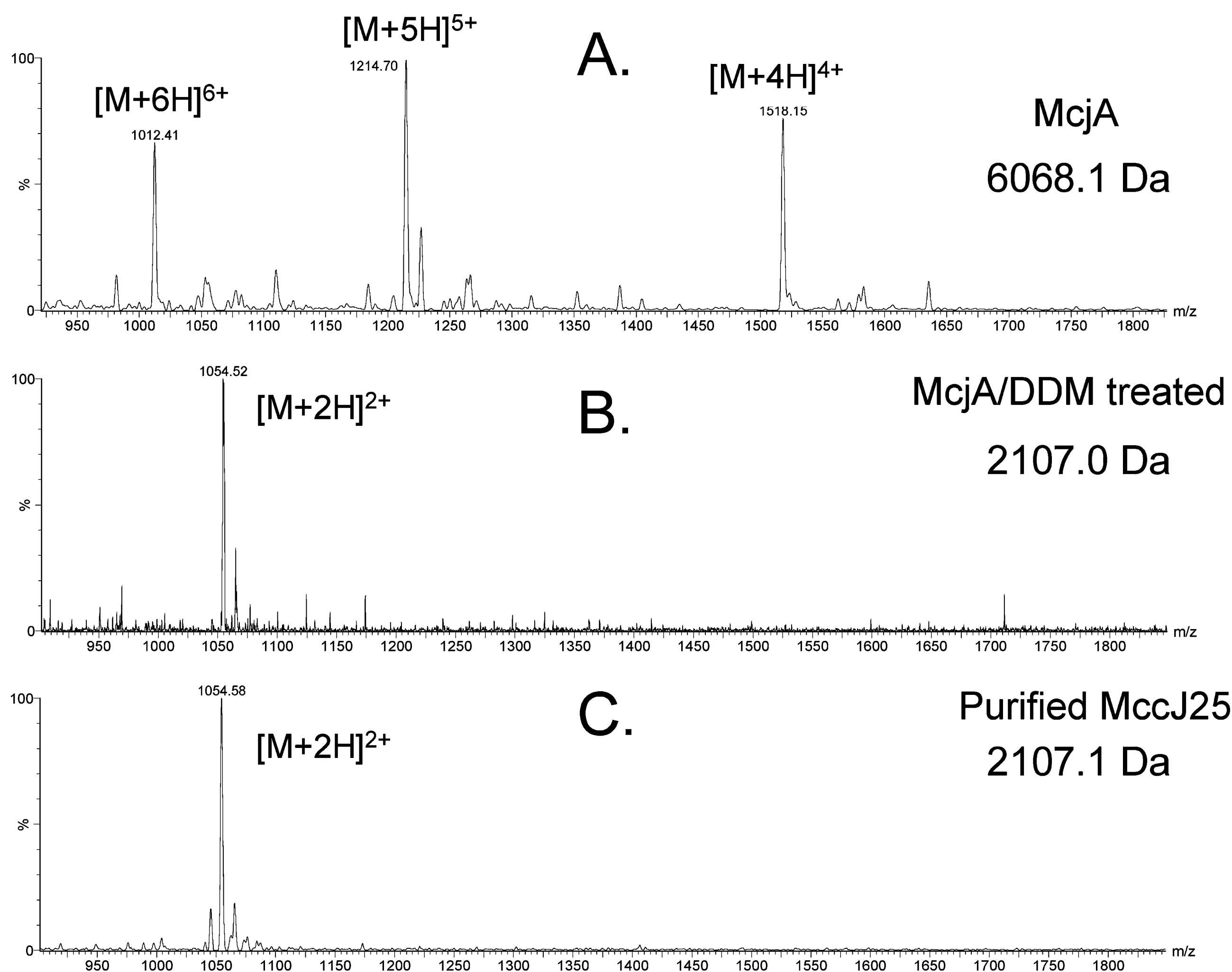


**Fig. 3** Mass spectrometry analysis of McjA maturation *in vitro*. Recombinant McjA precursor peptide displays a mass of 6068.1 Da, consistent with its predicted mass (Fig. 3A). Upon incubation with the DDM membrane extraction of *pTUC203C6H* expressing cells a thermostable species of mass 2107.0 Da was observed (Fig. 3B). This species has a mass consistent with mature, purified and active MccJ25 isolated from expressing cells containing *pTUC202* (2107.1, Fig. 3C).

The remaining glassy membranous material (containing inner and outer membranes) was resuspended in aqueous buffer prior to solubilisation. This was performed by treating the membrane suspension with two detergents (1% dodecyl-β-D-maltoside, DDM or 0.5% *N*-lauroyl sarcosine, NLS)‡. Histidine tagged McjC was detected in membrane fractions using Western blot (see ESI†). Insoluble debris was removed by ultracentrifugation and each detergent-soluble extract, as well as the original soluble lysate, was tested for its ability to mature recombinant McjA precursor peptide into MccJ25§. This was carried out by treating recombinant McjA (50 μg, which is inactive) with each extract at room temperature for four hours. The reaction was terminated by heating the mixture to 80 °C for 10 minutes and the resulting precipitate was removed by centrifugation. The remaining soluble fraction was tested for antimicrobial activity using a spot-on-lawn assay (Fig. 2B). Activity was clearly observed in the McjA samples that had been incubated with the DDM and NSL extracts. In contrast, no antimicrobial activity was detected in the sample containing McjA treated with the soluble protein extract prepared from the *pTUC203C6H* MccJ25-expressing cells. The conversion of McjA into MccJ25 was also monitored by mass spectrometry (Fig. 3). Analysis of McjA (observed mass 6068.1 ± 0.5 Da, Fig. 3A) after treatment with the membrane protein fraction of *pTUC203C6H* expressing cells clearly shows the presence of MccJ25 (observed mass 2107.0 ± 0.5 Da, Fig. 3B) which is identical to that observed in the isolated MccJ25 (Fig. 3C, see ESI†).

Amino acid sequence analysis of McjC predicts a protein that exhibits modest sequence similarity to both NAD-synthase and asparagine synthase—two ATP-dependant enzymes which catalyse the activation of a specific carboxyl group as an adenylate, followed by amide bond formation by reaction with an amine. This suggests that the formation of the amide bond required for lariat formation in MccJ25 biosynthesis may be ATP dependent. Indeed, we found that McjA maturation was greatly decreased if ATP was omitted from the membrane extraction buffer and Western blot analysis revealed that ATP was required for efficient extraction of McjC from the bacterial membrane (data not shown). Bioinformatic analysis (BLAST) with McjB identifies a small number of homologs, none of which currently have a known function.

Membrane protein extraction using NLS is specific for inner membrane proteins. Since extracts using this detergent can convert the mcjA precursor to the active microcin our results suggest that both McjB and McjC are located in the inner membrane fraction. Bioinformatic analysis of the MccJ25 exporter, McjD, predicts this protein to contain a cytoplasmic domain and several transmembrane regions, suggesting that it is located in the bacterial membrane. In contrast, no such membrane-spanning motifs are found in McjB and McjC. It is possible that McjB and McjC are membrane *associated*, perhaps in complex with the cytoplasmic domain of McjD. This would allow efficient shuttling of the mature MccJ25 from a McjB–McjC complex to the exporter, thus reducing the amount of potentially harmful free microcin in the cytoplasm. With the tools described in this work, we are now currently investigating this hypothesis.

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## Notes and references

‡ DDM extraction buffer: 50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM ATP, 0.5 mM EDTA, 1% dodecyl-β-D-maltoside, pH 8.0. NSL extraction buffer: 50 mM Tris, 150 mM NaCl, 1 mM DTT, 1 mM ATP, 0.5 mM EDTA, 0.5% *N*-lauroyl sarcosine, pH 8.0. After ultracentrifugation, membrane proteins were resuspended in a minimum volume of 50 mM Tris pH 7.5 and stored at −20 °C. Extraction of membrane proteins was achieved by addition of extraction buffer to a final total protein concentration of 6 mg $ml^{-1}$, and incubation at 4 °C for 1–18 hours.

§ Details concerning the expression and purification of recombinant McjA are provided in the ESI.†


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