RESEARCH LETTER

The Ile[13] residue of microcin J25 is essential for recognition by the receptor FhuA, but not by the inner membrane transporter SbmA

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Abstract

Entry of the peptide antibiotic microcin J25 (MccJ25) into target cells is mediated by the outer membrane receptor FhuA and the inner membrane protein SbmA. The latter also transports MccB17 into the cell cytoplasm. Comparison of MccJ25 and MccB17 revealed a tetrapeptide sequence (VGIG) common to both antibiotics. We speculated that this structural feature in MccJ25 could be a motif recognized by SbmA. To test this hypothesis, we used a MccJ25 variant in which the isoleucine in VGIG (position 13 in the MccJ25 sequence) was replaced by lysine (I13K). In experiments in which the FhuA receptor was bypassed, the substituted microcin showed an inhibitory activity similar to that of the wild-type peptide. Moreover, MccJ25 interfered with colicin M uptake by FhuA in a competition assay, while the I13K mutant did not. From these results, we propose that the Ile[13] residue is only required for interaction with FhuA, and that VGIG is not a major recognition element by SbmA.

Introduction

Microcin J25 (MccJ25) is a plasmid-encoded, 21-residue (2107 Da) peptide antibiotic excreted by a human intestinal isolate of *Escherichia coli* (Salomón & Farías, 1992; Blond *et al.*, 1999). *Escherichia coli* RNA polymerase (RNAP) is the target of antibiotic action (Delgado *et al.*, 2001; Yuzenkova *et al.*, 2002). Several pieces of evidence strongly suggest that MccJ25 binds to and clogs the secondary channel of the enzyme, which is supposed to be a conduit by which the nucleotide substrates reach the catalytic site. This would explain the inhibition of transcription by MccJ25 (Adelman *et al.*, 2004; Mukhopadhyay *et al.*, 2004).

Using a genetic approach, we demonstrated that MccJ25 uptake into *E. coli* is dependent on the outer membrane receptor FhuA (Salomón & Farías, 1993) and the inner-membrane proteins TonB, ExbB, ExbD, and SbmA (Salomón & Farías, 1995). Later, Destoumieux-Garzón *et al.* (2005) obtained biochemical evidence confirming the role of FhuA as the MccJ25 receptor. FhuA is a multifunctional protein that also serves as a receptor for colicin M (colM) and the phages T1, T5, and Φ80 and transports multiple substrates (iron-ferrichrome, and the antibiotics albomycin and rifamycin CGP4832) (Braun *et al.*, 1973; Hantke & Braun, 1975; Wayne & Neilands, 1975).

Four plasmid genes, *mcjA*, *mcjB*, *mcjC*, and *mcjD*, are required for MccJ25 production (Solbiati *et al.*, 1996; Solbiati *et al.*, 1999). The MccJ25 immunity protein, McjD, is an ABC exporter that, acting in concert with the outer-membrane channel TolC (Delgado *et al.*, 1999), actively expels the peptide, which would maintain its intracellular concentration below a toxic level. The *mcjA* structural gene encodes a 58-amino acid-long MccJ25 precursor, which is processed by the products of the *mcjB* and *mcjC* genes (Duquesne *et al.*, 2007) to yield the 21-residue mature peptide (G^{1}-G-A-G-H^{5}-V-P-E-Y-F^{10}-V-G-I-G-T^{15}-P-I-S-F-Y^{20}-G). The latter shows

an unusual threaded lasso structure, resulting from a cyclization between N-terminal residues Gly^{1} and Glu^{8} by lactamization, while the remaining tail (residues 9–21) loops back so as the C-terminal end penetrates the octapeptide ring, where it remains sterically grasped (Bayro *et al.*, 2003; Rosengren *et al.*, 2003; Wilson *et al.*, 2003). Residues F^{10}-V-G-I-G-T-P^{16} form a β-hairpin structure. Although MccJ25 is exceptionally resistant to most proteases, the peptide bond between Phe^{10} and Val^{11} is sensitive to thermolysin (Blond *et al.*, 1999). Digestion by this protease leads to a derivative of MccJ25, referred to as t-MccJ25, which consists of two peptide chains that remain associated due to topological trapping of the tail inside the ring (Rosengren *et al.*, 2004). As a consequence of this breakage, the tridimensional structure of the Val^{11}–Pro^{16} region is lost, while the structure of the ring and the threading C-terminal tail is conserved. The t-MccJ25 variant is unable to bind to FhuA *in vitro* and lacks antibacterial activity, but, as demonstrated by Bellomio *et al.* (2004) and Semenova *et al.* (2005), is still able to inhibit RNAP *in vitro*. This led to the notion that the ring-tail part of the molecule interacts with the target and that the β-hairpin loop is involved in reception and translocation by FhuA. Additional supporting evidence that the Val^{11} to Pro^{16} residues are important for MccJ25 uptake emerged from an extensive mutational scanning analysis of the molecule by Pavlova *et al.* (2008).

MccB17 is another peptide toxin dependent on SbmA for passage across the cytoplasmic membrane (Laviña *et al.*, 1986), but it does not use FhuA as a receptor. Although MccJ25 and MccB17 are structurally dissimilar, on comparing their sequences, we found that a tetrapeptide in the N-terminal end of MccB17, VGIG, also occurs in the interior of the β-hairpin region of MccJ25 (F^{10}-V-G-I-G-T-P^{16}). This prompted us to investigate whether this common structural feature is a recognition element by SbmA. If so, the loop region would be important for interaction not only with FhuA but also with SbmA. To address this question, in the present study, we have selected one of the MccJ25 mutants isolated previously, I13K, in which the Ile residue at position 13 was changed to Lys (Pavlova *et al.*, 2008). Based mainly on the results of osmotic shock experiments that allowed to bypass the FhuA-dependent step in MccJ25 uptake, we conclude that the substitution only affects translocation by FhuA, and that the I13K variant is properly recognized by SbmA.

Materials and methods

Culture media and growth conditions

Luria broth (LB)-rich medium and M9 minimal salts were purchased from Sigma Chemical Co. Minimal medium was supplemented with glucose (0.2%) and thiamine (1 $\mu g\,mL^{-1}$). Solid media contained 1.5% agar. Liquid cultures were grown with aeration by gyrotory shaking. Growth was monitored by measuring the $OD_{600\,nm}$. All cultures were incubated at 37 °C.

Purification of wild-type MccJ25 and I13K

The MccJ25 derivative I13K was generated in a previous work (Pavlova *et al.*, 2008). Plasmid pTUC202, a pACYC184 derivative carrying the entire MccJ25 operon (Solbiati *et al.*, 1996), was used as a template for PCR site-directed mutagenesis of the MccJ25-coding region of *mcjA* to obtain the desired substitution. The plasmid encoding the mutant microcin will be designated hereafter as pI13K. Wild-type and variant microcins were purified by a previously described procedure (Blond *et al.*, 1999). The starting materials were 4 L stationary-phase cultures in M9 medium of *E. coli* strain SBG231 transformed with plasmids pTUC202 or pI13K.

Microcin activity test

The minimum inhibitory concentrations (MIC) of MccJ25 and its analogue I13K were determined using a spot-on-lawn test, as follows: doubling dilutions of pure microcin preparations (1 $mg\,mL^{-1}$), in distilled water, were spotted (10 μL) onto LB plates and dried. Fifty microliters of a stationary-phase culture of the indicator strain were mixed with 3 mL of molten top agar (0.7% agar) and overlaid onto the plates. After overnight incubation, they were examined for different degrees of inhibition. The MIC of microcin was taken as the concentration (μM) of the last dilution yielding a clear or a turbid spot.

Osmotic shock treatment

For outer-membrane permeabilization, the phosphate-washing procedure of Cavard & Lazdunski (1981) was used. *Escherichia coli* AB1133 *tolC*::Tn*10* cells from a stationary-phase culture in LB medium were harvested by centrifugation, washed three times with 10 mM sodium phosphate buffer, pH 6.8, and resuspended to the desired concentration (about 2000 cells mL^{-1}) in the same buffer. Cell survival was evaluated in the absence or in the presence of either MccJ25 or I13K (both at a final concentration of 38 μM). After a 2-h incubation at 37 °C, 100-μL aliquots were plated in duplicate onto LB medium. After overnight incubation, the number of CFUs was recorded.

Results and discussion

Purification of the I13K variant

In the structure–function study of MccJ25 by Pavlova *et al.* (2008), the antibiotic activity assays of mutant microcins were performed with crude supernatants and, therefore, the results depended on the relative amounts of the particular derivatives in the supernatants. For a more accurate

comparison of the efficacy of entry of I13K with that of wild-type MccJ25 and for other experiments described below, we started by purifying both peptides to homogeneity by reversed-phase HPLC. Our first purification attempts were made using cultures in M9 minimal medium of strain DH5α harboring either plasmid pTUC202 or pI13K (encoding the wild-type or the mutant MccJ25, respectively). However, the plasmid encoding the mutant gene affected the growth efficiency of this strain (growth stopped at an $OD_{600\,nm}$ of 0.2–0.3), while the plasmid coding for wild-type MccJ25 did not. This phenotype was not seen in LB-rich medium, where the two strains grew to similar levels. A possible explanation for the growth deficiency phenotype could be that the mutant peptide was not being properly recognized by McjD, the dedicated microcin exporter, leading to an intracellular accumulation of the antibiotic above toxic levels. This was supported by the fact that *E. coli* strain SBG231 (which harbors an MccJ25-resistant mutant RNAP) (Delgado *et al.*, 2001) grew normally with either plasmid, suggesting that the RNAP mutation overcame the toxicity of accumulated microcin. The influence of the culture medium may be explained by the fact that the production of MccJ25 is higher in minimal medium (Salomón & Farías, 1992). Therefore, strain SBG231 was routinely used as a host to purify both native and mutant microcins. The yield of the purified mutant peptide was half that of native microcin (6–8 vs. 15–16 mg, respectively), which may also be explained by a deficient export to the extracellular medium.

Antibacterial activities of wild-type and mutant microcins

The substituted I13K derivative have been previously shown to retain the capacity for inhibition of RNAP *in vitro*, and so the loss of inhibitory activity was ascribed to a decreased efficiency of entry into bacterial cells (Pavlova *et al.*, 2008). To compare the antibacterial activities of purified MccJ25 and I13K, we used a spot-on-lawn test, as described in Materials and methods. As shown in Table 1, the mutant peptide was 32-fold less active on strain MC4100 than wild-type MccJ25. We have previously shown that *yojI*, an *E. coli* ORF with an unknown function, encodes an efflux pump that can use MccJ25 as a substrate (Delgado *et al.*, 2005). In fact, YojI seems to be responsible for part of the intrinsic resistance shown by most *E. coli* strains to MccJ25 (Socías *et al.*, 2009). Although the most likely explanation for the low activity of I13K, as compared with that of native MccJ25, would be a deficient uptake of the antibiotic, we investigated the possibility that both peptides enter cells to the same extent, but, once in the cytoplasm, the mutant peptide could be more efficiently pumped out than MccJ25 by YojI. In other words, the mutation could convert the peptide into a better substrate for export. If this hypothesis is correct, one would predict that in a *yojI* null background, the MIC of I13K should approach that of wild-type MccJ25. This prediction was not fulfilled, because when the experiment was repeated with strain MC4100Δ*yojI* as an indicator, the sensitivity to wild-type and mutant microcins was affected to the same extent (a twofold reduction in the MICs) (Table 1, second row).

The degree of susceptibility to MccJ25 is determined by the copy number of the components of the import machinery. Overexpression of FhuA from the multicopy plasmid pGC01 (pBR322 with *fhuA* cloned) results in an increased sensitivity to the antibiotic. This is also seen with the substituted MccJ25 derivative, as illustrated by the stab test shown in Fig. 1. The zones of inhibition formed by the native and mutant microcins in plates seeded with the FhuA-overproducing strain MC4100 (pGC01) were larger than those formed on a lawn of the control strain MC4100. This was particularly noticeable for the I13K variant. It was almost undetectable when MC4100 was used as an indicator, whereas a much larger halo appeared on the FhuA overproducer. For a more quantitative comparison, we measured the MICs of MccJ25 and I13K for MC4100 (pGC01). As can be seen in Table 1, overexpression of FhuA results in a markedly increased (eightfold) susceptibility to MccJ25, as compared with that of the control MC4100. Upon overexpression of FhuA, the MIC

Table 1. MICs (μM) of native MccJ25 and its analogue I13K for some *Escherichia coli* strains used in this study*

Strain	MccJ25	I13K
MC4100	1.95	62.50
MC4100 Δ*yojI*	0.97	31.25
MC4100 (pGC01)	0.24	0.98

*MICs were determined by a spot-on-lawn test as indicated in the text.

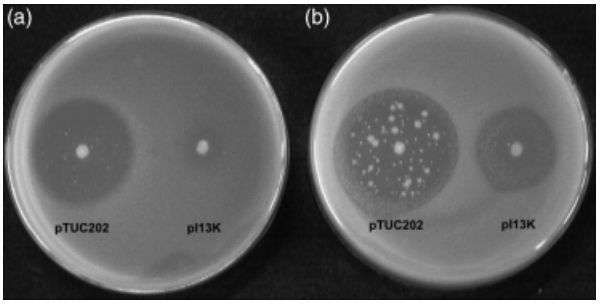

Fig. 1. Effect of FhuA receptor overproduction on the sensitivity to MccJ25 and its analogue I13K. Colonies of strain SBG231 transformed with either pI13K or pTUC202 were stabbed into plates of M9 minimal medium plates and pregrown overnight at 37 °C before exposure to chloroform vapors for 15 min. After allowing residual chloroform to evaporate for 1 h, the plates were overlaid with a lawn of (a) MC4100 and (b) MC4100 (pGC01), which overproduces the FhuA receptor. The plates were then incubated overnight at 37 °C.

of I13K was considerably reduced (64-fold), approaching that of native MccJ25. This suggests that the defective step for I13K importation is at the FhuA level, and that the increased numbers of receptors compensate for this defect. Alternatively, SbmA could be the limiting step, but the increased concentration of I13K molecules in the periplasmic space resulting from FhuA overexpression might allow more rapid entry through SbmA.

Bypass experiments

To clarify which of the steps in the microcin-uptake pathway is responsible for the the decreased entry of I13K into the cells, we bypassed the requirement for FhuA using the mild osmotic shock procedure of Cavard & Lazdunski (1981) (see Materials and methods). To increase the sensitivity of the assay, we used *E. coli* AB1133 *tolC*::Tn*10* as the tester strain (TolC-deficient strains are hypersusceptible to MccJ25; Delgado *et al.*, 1999). The results are shown in Table 2. The osmotic shock *per se* did not lead to any decrease in cell viability. As expected, in the absence of a functional FhuA receptor, there was no effect of MccJ25, whereas following osmotic shock, the viable count decreased to 40% of the initial number of cells. The fact that an FhuA receptorless MccJ25-resistant strain became sensitive upon osmotic shock indicates that the treatment is effective to allow the entry of MccJ25 in an FhuA-independent manner. Using the same procedure, Cavard & Lazdunski (1981) reported that colicin A, a 63-kDa protein, can be shocked with high efficiency into *E. coli* cells devoid of the colicin receptor. In spite of the small size of MccJ25 (2 kDa) relative to colicin A, which would be expected to allow it an easier access to the periplasm, the amount of peptide that can be osmotically shocked into cells is limited. This can be seen from the number of shocked FhuA-defective cells killed (60%), which is significantly lower than the 100% of cells killed when FhuA is present during the shock. Perhaps there is some structural feature of the microcin molecule that slows down its passage through the outer-membrane bilayer. When I13K was added to Δ*fhuA* cells without shock, it caused no detectable loss of viability, but was able to reduce the number of viable cells to 44% of the initial input when osmotically shocked into the periplasm. Therefore, the MccJ25 variant was almost as active as wild-type MccJ25 after the phosphate-washing treatment, indicating that once FhuA is bypassed, both peptides are similarly recognized and transported by SbmA.

Table 2. Action of wild-type MccJ25 and I13K variant with and without osmotic shock*

	Cells surviving (% of input)		
Strain and treatment	No added microcin	MccJ25	I13K
AB1133 *tolC*::Tn*10* without shock	100	0	100
AB1133 *tolC*::Tn*10* shocked	100	0	35
AB1133 *tolC*::Tn*10* Δ*fhuA* without shock	100	100	100
AB1133 *tolC*::Tn*10* Δ*fhuA* shocked	100	40	44

*Cells were grown and subjected to osmotic shock as described in the text. After shock, appropriate dilutions were plated onto LB plates. A value of 100% corresponds to 2100–2300 viable cells mL^{-1}. Values are averages of two independent experiments.

One possible explanation for the considerably reduced biological activity of I13K might be that the substitution of Ile^{13} for Lys renders it susceptible to degradation by an intracellular protease. However, the fact that upon shocking into the periplasm its activity is comparable to that of wild-type MccJ25 makes this an unlikely explanation. It may be argued that the activity defect of the I13K variant might result not from impairment of the function of the residue modified, but because the large size of the Lys side chain introduced could create a steric blockade that impedes proper interactions of neighboring groups with FhuA. However, it is of interest to note that replacement of Ile^{13} with Arg, which also has a bulky and positively charged side chain, does not reduce, but rather enhances the potency of the mutated peptide as compared with that of wild-type MccJ25 (Pavlova *et al.*, 2008). All in all, the results shown until now indicate that the reduced antibiotic activity of I13K results from its impaired ability to be taken up by FhuA.

Native MccJ25, but not I13K, interferes with colM uptake by FhuA

ColM shares the FhuA receptor with MccJ25. To provide further support for the notion of defective recognition of I13K by FhuA, we performed competition assays between colM, MccJ25, and I13K. To obtain a crude preparation of colM, an exponential-phase culture of the colicinogenic strain TO4 (Braun *et al.*, 1980) in LB was induced with mitomycin C. Following an additional 2-h incubation, cells were spun down and the supernatant fluid was sterilized by filtration and used as a source of colicin. ColM was diluted 1 : 50 in solutions of MccJ25 or I13K in distilled water, both at 1 mg mL^{-1}, to ensure an excess of peptides. Samples of the mixtures were spotted on an LB plate seeded with strain SBG231 as an indicator. It was necessary to use this strain, which is fully resistant to MccJ25, but susceptible to colM, to detect only the effect of the latter. As can be seen in Fig. 2, native MccJ25 almost completely blocked colM activity. Because translocation by FhuA is the only step shared by MccJ25 and colM in their mechanisms of action, MccJ25-mediated protection from killing by colM was most likely due to specific competition for receptor binding. In contrast, no protection was afforded by the I13K variant,

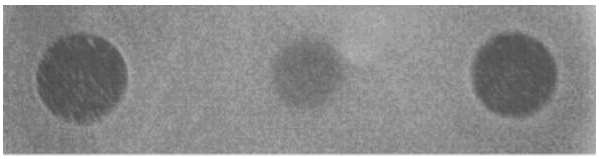

Fig. 2. *In vivo* competition between colM, MccJ25, and I13K for binding to FhuA. From left to right: colM diluted 1 : 50 in distilled water, colM diluted 1 : 50 in a 1 mg mL^{-1} solution of MccJ25, and colM diluted 1 : 50 in a 1 mg mL^{-1} solution of the I13K analogue. Ten-microliter drops of each dilution were spotted on an M9 plate and allowed to dry. The plate was then overlaid with soft agar containing SBG231 cells. Note that only MccJ25 was able to protect *Escherichia coli* cells from colM killing, whereas I13K had no inhibitory effect on colM-binding activity.

suggesting that it was unable to prevent the reception or translocation of colM by FhuA. This is further evidence that the mutated microcin is poorly recognized by FhuA.

Summing up, the findings presented in this work are consistent with the conclusion that the Ile^{13} residue in the β-hairpin loop of MccJ25 is required for interaction with FhuA, but not for transport through the inner-membrane protein SbmA. This is the first report in which a purified MccJ25 variant in the loop region has been used to define the role of a particular residue in the interaction with the components of the import machinery. In addition, our results strongly suggest that the putative VGIG motif in this part of the MccJ25 molecule is not a major recognition element by SbmA.

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