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Parasitism of iron-siderophore receptors of *Escherichia coli* by the siderophore-peptide microcin E492m and its unmodified counterpart

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Abstract

Microcin E492 (MccE492) is an antibacterial peptide naturally secreted by *Klebsiella pneumoniae* RYC492. Initially described as an 84-residue unmodified peptide, it was also recently isolated in a posttranslationally modified form, MccE492m. The production of MccE492m is dependent on the synthesis of enterobactin and the *mceABCDEFGHIJ* gene cluster. The posttranslational modification was characterized as a trimer of *N*-(2,3-dihydroxybenzoyl)-L-serine (DHBS) linked to the Ser84-carboxylate *via* a β-D-glucose moiety. MccE492m was shown to bind ferric ions through the trimer of DHBS. This is the first example of a novel type of antibacterial peptide termed siderophore-peptide. Recognition of MccE492m, but also of the unmodified MccE492, was shown to be mediated by the catecholate siderophore receptors FepA, Cir and Fiu at the outer membrane of *E. coli*. The siderophore-type modification was shown to be responsible for a significant enhancement of the microcin antibacterial activity. Therefore, we propose that MccE492 and MccE492m use iron-siderophore receptors for uptake into the target bacteria and that improvement of MccE492 antimicrobial activity upon modification results from an increase in the microcin/receptor affinity.

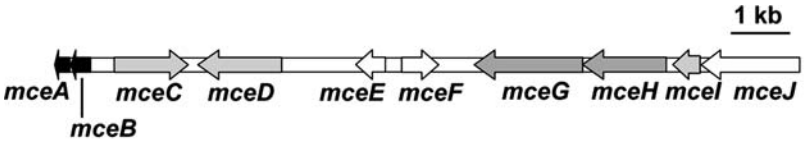
Introduction

Microcins are gene-encoded, low-molecular-weight antibacterial peptides produced by *Enterobacteriaceae*, mostly *Escherichia coli* (Baquero & Moreno 1984). They inhibit the growth of phylogenetically related species and are believed to take part in the control of microbial populations within the intestinal tract. Microcins display a broad diversity of structures and mechanisms of action but a conserved general organization of their genetic systems. The gene clusters typically include open reading frames encoding the microcin precursor, modification enzymes, secretion factors and immunity proteins (Braun *et al.* 2002; Moreno *et al.* 2002). In this review, we have focused on the structure, antibacterial activity, and recognition/translocation systems of microcin E492 with the aim of showing how the recently characterized siderophore-type posttranslational modification it displays provides new clues for understanding the peptide biosynthesis and uptake into target bacteria. The existence of a family of siderophore-peptides including microcins E492, M and H47 is discussed.

Genetic and structural features

The MccE492-encoding genetic system

Contrary to most of the microcins, for which the required genes are plasmid-encoded, the genetic determinants involved in MccE492 production, export and immunity (*mceABCDEFGHIJ*) are located in the bacterial chromosome of *Klebsiella pneumoniae* RYC492 (Wilkens *et al*. 1997). The cloning of the MccE492-encoding system leading to the construction of the pJAM229 plasmid (Wilkens *et al*. 1997) was a major step forward, which not only allowed the characterization of the MccE492 gene cluster but also facilitated the production and isolation of the microcin. The function of only 5 out of the 10 genes required for the production and secretion of mature MccE492 has been identified (Figure 1). Thus, *mceA* and *mceB* encode the microcin precursor and immunity protein, respectively, while *mceG*, *mceH*, and presumably *mceF* are responsible for microcin export. On the basis of sequence homology, an enzymatic activity was speculated for the product of 3 other genes: glycosyltransferase (*mceC*), acyltranferase (*mceI*) and enterobactin esterase (*mceD*), while no



Gene	Homology with known proteins (accession no.)	Identified[a] or hypothetical[b] function	Reference
mceA	-	Precursor[a]	Wilkens *et al.*1997 Lagos *et al.* 1999
mceB	-	Immunity[a]	
mceC	IroB C-glycosyltransferase (TrEMBL: Q8GH22)	Maturation[b] (enterobactin C-glycosylation)	Lagos *et al.* 2001 Thomas *et al.* 2004 This article
mceD	IroD enterobactin esterase (TrEMBL: Q8GH20)	Maturation [b] (enterobactin cleavage)	Thomas *et al.* 2004 This article
mceE	-	unknown	-
mceF	-	Export[a]	Castillo JA & Lagos R (unpublished) see Lagos *et al.* 2001
mceG	ABC transporter (SWISSPROT: P22520)	Export[a]	
mceH	Secretion protein (SWISSPROT: P22519)	Export[a]	
mceI	Acyltransferase LktC (SWISSPROT: P16461)	Maturation [b]	Lagos *et al.* 2001 Thomas *et al.* 2004
mceJ	-	Maturation [b]	Solo & Lagos R (unpublished) see Lagos *et al.* 2001

Figure 1. Genetic system responsible for MccE492 synthesis, export and for the immunity of the producing strain to the microcin. Five out of the 10 genes composing the MccE492 gene cluster have been shown to encode the precursor (*mceA*), an immunity protein (*mceB*) and an ABC protein complex (*mceG*, *mceH*) involved in the microcin export, with a potential accessory role for *mceF*. The function of *mceC*, *mceD*, *mceE*, *mceI*, and *mceJ* remains hypothetical or unknown. Genes are shown proportional to their size.

sequence similarities were found for *mceJ* and *mceE* (Lagos *et al*. 2001).

The absence of posttranslational modification in the MccE492 initially described (Pons *et al*. 2002), together with the finding that inactivation of the *mceC*, *mceI* or *mceJ* genes resulted in the loss of the MccE492 antibacterial activity without any apparent modification of the primary structure (Corsini *et al*. 2002) raised many questions on the function of these genes, which were consequently proposed to encode chaperone proteins (Corsini *et al*. 2002). The recent finding that MccE492 can be synthesized in a posttranslationally modified form provides the *mceC*, *mceD and mceI* genes with a very likely role in the acquisition of the identified posttranslational modification (Thomas *et al*. 2004). The proposed functions, reported in Figure 1, are discussed below.

Biosynthesis of MccE492 as a siderophore-peptide

Recently, a new peptide displaying both a higher molecular mass (831 Da mass increase) and an enhanced specific activity (see following section) compared to MccE492 was isolated from culture supernatant of *E. coli* VCS257 pJAM229 (Thomas *et al*. 2004). The new peptide, which we named MccE492m, was a modified form of MccE492. Comparison of the chymotrypsin digest of the modified and unmodified microcins provided the localization of the posttranslational modification on a short peptide termed MccE492m[74–84] corresponding to the 11-residue C-terminal region of the microcin. Complete determination of the MccE492m[74–84] structure was obtained by the combined use of Collision Induced Dissociation (CID) experiments performed by Electrospray Ionization Ion Trap Mass Spectrometry (ESI-IT-MS) in the positive and negative modes and by high field (800 MHz) two-dimensional $^{1}H/^{13}C$ Nuclear Magnetic Resonance (NMR) spectroscopy. A large body of evidence demonstrated that the modification consists of a linear trimer of *N*-(2,3-dihydroxybenzoyl)-L-serine (DHBS) linked to the Ser84 carboxylate through a β-D-glucose moiety, itself linked to the first DHBS unit through a *C*-glycosidic bond and to the microcin Ser84 carboxylate through an *O*-glycosidic bond (Figure 2) (Thomas *et al*. 2004).

The structure of this novel posttranslational modification is reminiscent of siderophores. These molecules employ hydroxamates, catecholates or α-hydroxycarboxylates to chelate ferric iron (Fe^{III}), which they make available for bacterial consumption (Braun & Braun 2002; Andrews *et al*. 2003). One major siderophore involved in iron uptake in

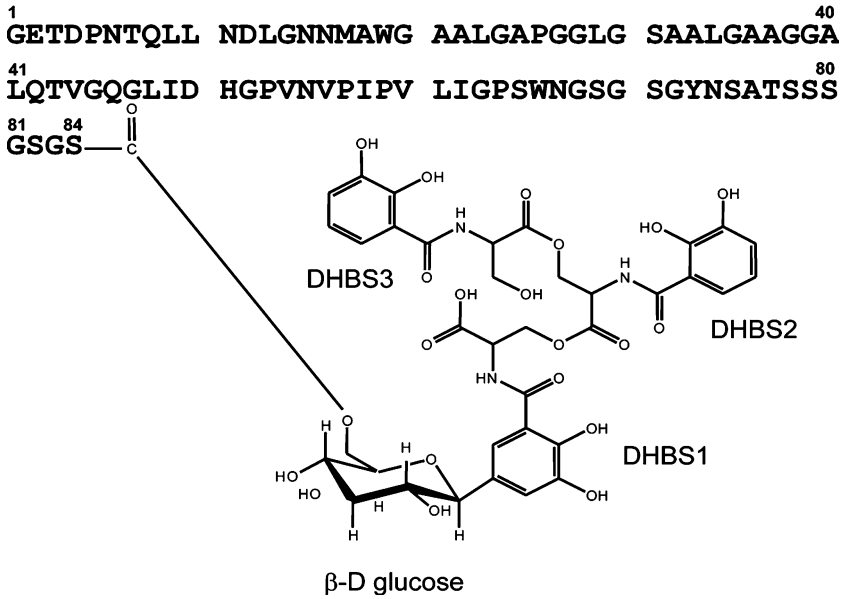


Figure 2. Structure of MccE492m. The posttranslational modification carried by Ser84 is composed of a β-D glucose linked to 3 DHBS units.

E. coli is the catecholate enterobactin, a cyclic trimer of DHBS. Together with its breakdown products, the linear DHBS trimer, dimer and monomer, enterobactin is able to transport ferric ions into enterobacteria (Hantke 1990). Interestingly, parallel to the characterization of MccE492m structure in our group, Hantke and colleagues isolated novel catecholate siderophores from *Salmonella* termed salmochelins, which derive from enterobactin and carry β-D-glucose moieties linked to DHBS units through *C*-glycosidic bonds (Bister *et al*. 2004). The recent characterization of MccE492m structure (Thomas *et al*. 2004), together with the studies on salmochelin biosynthesis (Fischbach *et al*. 2005; Zhu *et al*. 2005), and the finding that MccE492m production requires enterobactin synthesis (Thomas *et al*. 2004) allow us to confirm and precise the roles we proposed earlier for MceC and MceD in the acquisition of the microcin modification. Thus, presenting 75.6% identity with IroB, an enterobactin *C*-glycosyltransferase involved in salmochelin biosynthesis (Fischbach *et al*. 2005), MceC is likely to catalyze the *C*-glycosylation of enterobactin observed in MccE492m posttranslational modification. Similarly, as shown for IroD (Zhu *et al*. 2005; Lin *et al*. 2005.), which is 57% identical to MceD, MceD could be involved in enterobactin degradation, i.e. opening of the cyclic trimer of DHBS to generate the linear DHBS trimer found in MccE492m (Figure 1).

The siderophore-like structure of MccE492m modification prompted us to investigate the specific iron-binding properties of the modified microcin (Thomas *et al*. 2004). Adding up to 8 equivalents of $FeCl_3$ to MccE492[74–84], the C-terminal fragment of unmodified MccE492, did not induce any change in the mass spectrum obtained by ESI-IT-MS in the positive mode (data not shown), whereas addition of 2 equivalents of $FeCl_3$ to MccE492m[74–84] led to a major species at m/z 913.3 (Figure 3a) that was assigned to $[(M\text{-}H) + Fe^{III}]^{2+}$ and was indicative of the specific binding of one Fe^{III} to the posttranslationally modified peptide. Furthermore, CID of the cationized ions allowed the localization of the metal-binding sites (Figure 3b). In the positive mode, the series of acylium-ions described the 74–84 sequence and was identical to that observed

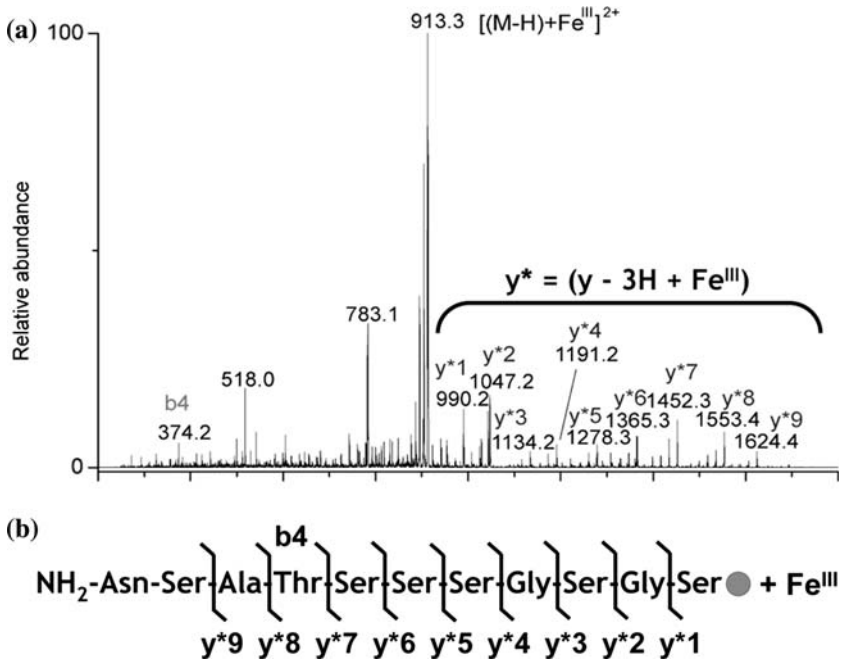


Figure 3. Characterization of MccE492m[74–84] iron-binding properties by ESI-IT-MS. Data were acquired in the positive mode in the presence of two equivalents $FeCl_3$. (a) CID of the cationized $[(M\text{-}H) + Fe^{III}]^{2+}$ species. The y-type ion series is shifted by a m/z ratio consistent with a 52.9 Da mass increase, which raises the $y^* = (y\text{-}3H + Fe^{III})$ ion series. (b) Resulting MccE492m[74–84] fragmentation pattern. b and y ions observed on the CID spectrum are shown. MccE492m posttranslational modification is represented as a grey circle (●).

for the unmodified peptide, while the series of ammonium ions (y1–y9) showed a systematic mass increase indicative of the binding of one Fe^{III} to the MccE492m[74–84] C-terminus (Figure 3). Complementary CID in the negative mode confirmed a preferential binding of Fe^{III} to the region of MccE492m[74–84] that contains the DHBS units through their catecholate functionalities. Taking into account both the siderophore structure of the modification and its Fe^{III}-binding ability, MccE492m was assigned as a siderophore-peptide, thus building up a new type of antimicrobial peptide (Thomas *et al.* 2004).

Antibacterial activity and membrane-permeabilizing properties

Antibacterial activity

Studies on MccE492 and MccE492m antimicrobial properties showed that both peptides have a narrow-spectrum of activity, only some species of Gram-negative *Enterobacteriaceae* being susceptible in the range of 0–10 μM (Destoumieux-Garzón *et al.* 2003; Thomas *et al.* 2004). Interestingly, MccE492m was 2–8 times more active than MccE492 with minimum inhibitory concentrations (MICs) ranging from 40 to 160 nM against *E. coli* and *Salmonella enterica* strains. Furthermore, in the range of concentration tested, MccE492m activity extended to *Enterobacter cloacae* and *K. pneumonia*e strains with MIC values of 0.6 and 2.5 μM, respectively. The activity of both MccE492 and MccE492m was bactericidal with minimal bactericidal concentrations similar to MIC values (Destoumieux-Garzón *et al.* 2003; Thomas *et al.* 2004). The loss of viability of *E. coli* ML35 was very rapid for MccE492m with all bacteria killed within 3 min, while MccE492 required more than 30 min (Figure 4a). Similar results were obtained with *E. coli* W3110 (unpublished data). Therefore, acquisition of a siderophore-like posttranslational modification provides MccE492 with a higher specific activity in terms of antibacterial properties.

Membrane-permeabilizing activity

Early studies by de Lorenzo and Pugsley evidenced the presence of a substance able to depolarize the cytoplasmic membrane of *E. coli* in the secretions of an MccE492-producing strain (de Lorenzo & Pugsley 1985). Later, such bacterial secretions were shown to form ion-channels in planar lipid bilayers leading the authors to suggest that the membrane activity is responsible for MccE492 bactericidal effect (Lagos *et al.* 1993). Thanks to

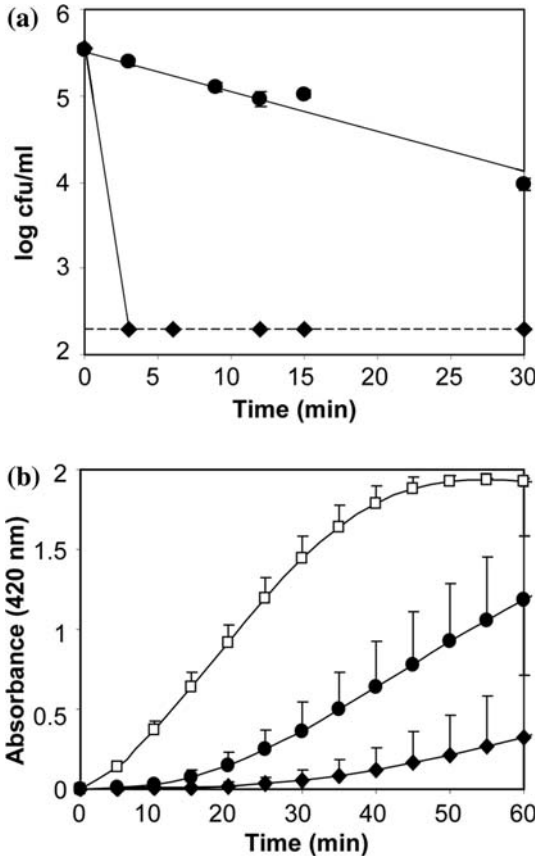


Figure 4. Kinetics of bacterial killing and of *in vivo* membrane permeabilization. (a) MccE492m (1.3 μM, ◆) or MccE492 (1.3 μM, ●) was added to an exponential phase culture of *E. coli* ML35. Aliquots were removed at different time intervals, diluted and plated on nutrient agar as described in (Destoumieux-Garzón *et al.* 2003). Surviving bacteria were counted as colony-forming units (cfu) after overnight incubation at 37 °C. Control in the absence of peptide indicated no spontaneous loss of viability. The reliable limit of detection (200 cells/ml) is indicated by a dashed line. Error bars represent standard deviations for three independent experiments. (b) Stationary growth phase *E. coli* ML35 were incubated for 1 h at 37 °C in 10 mM phosphate buffer pH 7.4 containing 0.1% (v/v) trypticase soy broth. MccE492 (1 μM, ●) or MccE492m (1 μM, ◆) were then added to the reaction with 625 μM ONPG. β-galactosidase activity was monitored spectrophotometrically at 420 nm. Melittin (0.05 μM, □) was used as a positive control. No significant ONPG hydrolysis was detected in the presence of buffer only. Results are representative of three independent experiments.

the availability of pure peptide preparations, the activity of MccE492 and MccE492m on the bacterial cytoplasmic membrane was reinvestigated. *E. coli* ML35, a strain that constitutively expresses a cytoplasmic β-galactosidase and is lactose permease deficient (Lehrer *et al.* 1988) was used for this purpose. Indeed, due to the strain deficiency in lactose transport, only damages to the cytoplasmic membrane can lead to hydrolysis of extracellular *o*-nitrophenyl β-D-galactopyranoside (ONPG), an impermeable chromogenic substrate of β-galactosidase. ONPG hydrolysis was observed when live bacteria were incubated with either MccE492 (Destoumieux-Garzón *et al.* 2003) or MccE492m (Figure 4b). Nevertheless, the time-course of hydrolysis was much slower for both microcins than for melittin, a pore-forming peptide from honeybee venom used as a positive control (Dempsey 1990). Unexpectedly, despite a more potent antibacterial activity against *E. coli* ML35, MccE492m induced cytoplasmic membrane permeabilization more slowly than MccE492 (Figure 4b). Consequently, the rapid decrease in cell viability observed with MccE492m is not directly correlated with a rapid permeabilization of the cytoplasmic membrane. This indicates that interference with the cytoplasmic membrane is not responsible by itself for the lethal effect of both microcins.

The activity of MccE492/MccE492m on the cytoplasmic membrane of *E. coli* prompted us to observe by electron microscopy the potential damages caused to the membranes by MccE492m. The microcin was used at lethal doses against the microcin-susceptible *E. coli* W3110 and the bacteria were fixed after a 1 h-incubation. Ultra-thin sections of the treated and untreated bacterial suspensions were then observed for morphological changes due to the microcin treatment. The first observation was that the overall integrity of the cells was preserved in microcin-killed bacteria. Indeed, general features such as size, shape and membranes were very similar in microcin-treated bacteria and controls (Figure 5a and b). This confirmed earlier results from the enzymatic ONPG diffusion assay, which indicated that MccE492 does not induce cell lysis (Destoumieux-Garzón *et al.* 2003). The major difference between treated and untreated cells was the swelling of the periplasmic space in microcin-killed bacteria (Figure 5a, c and e). We also barely observed any organized genetic material in treated cells, which conversely usually displayed dense cytoplasm (Figure 5a). At high magnification, the presence of both intact cytoplasmic and outer membranes was clearly observed (Figure 5e), indicating that permeabilization of the cytoplasmic membrane is a subtle mechanism that most likely rather compares with pore formation as observed in artificial membranes (Lagos *et al.* 1993; Destoumieux-Garzón *et al.* 2003), than with membrane solubilization.

Recognition/translocation

Membrane proteins involved in antibacterial activity

Initial studies on MccE492 mechanism of action had shown that many bacteria resistant to the secretions of MccE492-producing strains possessed mutations in the TonB-encoding gene (Pugsley *et al.* 1986). The TonB protein is involved in energy transduction from the cytoplasmic membrane, to which it is anchored, to several outer membrane receptors (Postle 1993; Braun 1995). TonB is also associated with the cytoplasmic membrane proteins ExbB and ExbD, thus forming the TonB system. Interestingly, group B colicins, antibacterial proteins secreted by *Enterobacteriaceae*, have parasitized iron-siderophore receptors coupled to the TonB energy transduction system for translocation across *E. coli* outer membrane (Lazdunski *et al.* 1998; Cao & Klebba 2002). We therefore made the hypothesis that TonB could be involved in a translocation event required for MccE492 mechanism of action. We first confirmed the role of TonB in the microcin activity by testing the antibacterial activity of a pure microcin preparation against isogenic strains mutated in the *tonB* gene. TonB was shown to be required for the antibacterial activity of both MccE492 (Destoumieux-Garzón *et al.* 2003) and MccE492m (Thomas *et al.* 2004). Indeed, the *tonB* mutation induced a high resistance to MccE492 and MccE492m and the antibacterial activity of both microcins was completely restored in the TonB-complemented strain. The role of TonB in microcin translocation was then assayed by measuring the bacterial cytoplasmic membrane potential before and after a 15-min treatment of various *E. coli* strains with lethal doses of MccE492 and MccE492m (Destoumieux-Garzón

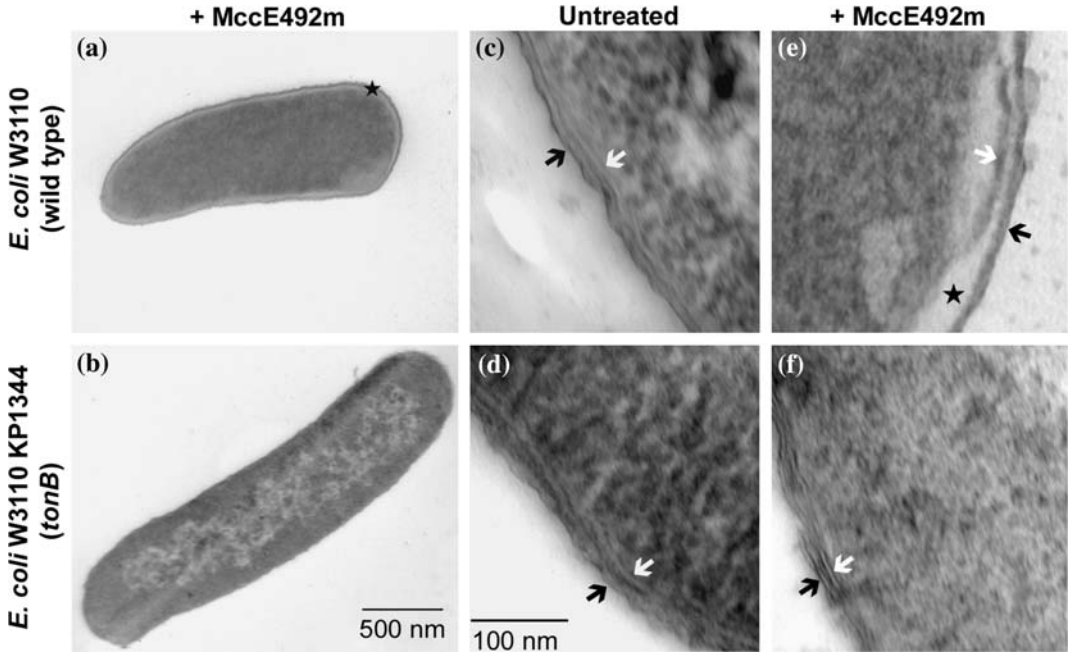


Figure 5. Morphology of microcin-treated *E. coli*. Wild-type *E. coli* W3110 (a, c, e) or *E. coli* W3110-KP1344 *tonB* (b, d, f) in exponential phase of growth were washed and resuspended in 1 ml of 0.1 M phosphate buffer pH 7.4 at 5×10^8 cells/ml. Cell suspensions were then incubated for 1 h with 5 μM MccE492m (a, e, b, f) or buffer only (untreated cells, c, d). Bacteria were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. After 30 min, 1% osmium and 0.1% ruthenium red were successively added. After 1 h at room temperature, the suspension was rinsed in phosphate buffer, dehydrated and embedded in Spurr Resin. Ultra-thin sections were poststained with uranyl acetate at 30 °C and observed with a H 710 Hitachi electron microscope under a 20.000×(a, b) or 150.000× magnification (c, d, e, f). White and black arrows are pointing at the cytoplasmic and outer membranes, respectively. A star indicates swollen periplasmic space in microcin-treated wild-type bacteria.

et al. 2003). As expected from our cytoplasmic membrane permeabilization assays (Figure 4b), MccE492 produced a rapid depolarization of the cytoplasmic membrane in the wild-type *E. coli* W3110 (Figure 6). This effect was not observed with the *E. coli* W3110-KP1344 *tonB* isogenic strain, but was restored when the mutant strain was complemented by a TonB-encoding plasmid (Figure 6). A similar effect was observed with MccE492m (unpublished data). Consistent with the membrane depolarization data, no difference could be observed at the ultrastructural level between microcin-treated and untreated *E. coli* W3110-KP1344 *tonB*. Cytoplasmic and outer membranes were as close as in untreated controls (Figure 5d and f) and organized genetic material was seen (Figure 5b). The requirement for TonB for both antibacterial activity and cytoplasmic membrane permeabilization strongly suggests that energy is required for the microcin mechanism of action. That was clearly shown by our bactericidal assays in the presence of carbonyl cyanide

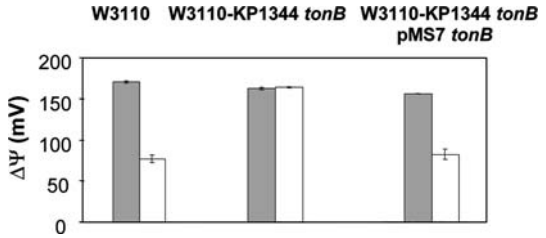


Figure 6. Microcin-induced cytoplasmic membrane depolarization of *E. coli*. A bacterial suspension of *E. coli* (5×10^8 cells/ml) loaded with [^{3}H]-labeled tetraphenyl phosphonium bromide, was incubated in the presence of 5 μM MccE492 (white bars) or 0.1 M phosphate buffer pH 7.8 (grey bars). After 15 min, the radioactivity associated to the bacteria was counted and the cytoplasmic membrane potential ($\Delta\psi$) was calculated according to the Nernst equation assuming an intracellular volume of 1 μl for 2×10^6 cells. The bacterial strains used are *E. coli* W3110 (wild-type), W3110-KP 1344 *tonB*, and W3110-KP 1344 *tonB* complemented by pMS7 *tonB*.

m-chlorophenylhydrazone (CCCP), an uncoupler of the cytoplasmic membrane proton gradient (Kaback *et al*. 1974), in which MccE492 antibacterial activity was shown to require the presence of

a proton motive force at the cytoplasmic membrane (Destoumieux-Garzón *et al.* 2003). Altogether, our results show that MccE492 and MccE492m are dependent on both TonB and energy for translocation across the outer membrane and subsequent antibacterial activity.

Together with the need for TonB, the narrow spectrum of bactericidal activity of both MccE492 and MccE492m, suggests that these peptides could interact with bacterial outer membrane receptor(s) to enter the target cell (Destoumieux-Garzón *et al.* 2003; Thomas *et al.* 2004). The siderophore-peptide structure of MccE492m prompted us to screen bacteria deficient in TonB-dependent iron-siderophore receptors for susceptibility to both MccE492 and MccE492m. Comparison of the microcin MICs against mutants in the outer-membrane receptors involved in the uptake of iron complexed with catecholate siderophores (FepA, Cir, and Fiu) led us to the conclusion that all three receptors are required for antibacterial activity of MccE492 and MccE492m (Table 1). In an earlier study, Hantke and colleagues had shown that a MccE492-producing strain was unable to inhibit the growth of a *fepA cir fiu* triple-mutant (Patzer *et al.* 2003). By using pure peptide preparations, we demonstrated that the *fepA cir fiu* triple-mutant was at least 200-fold more resistant to the microcins than the wild-type isogenic strain (Thomas *et al.* 2004). Interestingly, by using single, double and triple mutants deriving from the same isogenic strain, we demonstrated that FepA and Fiu were primarily involved in the recognition/translocation system, whereas Cir played a secondary role (Thomas *et al.* 2004). These results were confirmed by Strahsburger *et al.* (2005). Thus, MccE492/MccE492m are recognized and translocated across the outer membrane of *E. coli* by the receptors involved in the uptake of enterobactin and its breakdown products, the linear trimer, dimer, and monomer of DHBS, which are similarly imported through a TonB- and energy-dependent mechanism (for review see Braun & Braun 2002; Andrews *et al.* 2003). At this time, there is still no evidence of the direct interaction of MccE492/MccE492m with all three iron-siderophore receptors. However, the observation that (i) cyclic or linear trimers of DHBS are antagonist of MccE492 activity (Orellana & Lagos 1996; Strahsburger *et al.* 2005), and (ii) that MccE492m carries a linear trimer of DHBS as a posttranslational modification (Thomas *et al.* 2004), strongly suggests that catecholate siderophores compete with MccE492/MccE492m for binding to the outer membrane receptors FepA, Cir and Fiu. *In vitro* interaction studies should help us determining if there is a differential affinity of the microcin for its receptors that could be correlated to the MICs observed on the mutant strains. They should also clarify the role of the modification in the recognition mechanism. It is indeed very likely that acquisition of a siderophore-type modification is an efficient way for the microcin to improve its uptake by the target bacteria, by gaining affinity for its receptors.

Table 1. Antibacterial activity of MccE492 and MccE492m against wild-type and isogenic mutant strains displaying impaired iron-uptake systems.

Bacterial strain	MIC (μM)	
	MccE492	MccE492m
E. coli MC4100	0.32	0.16
E. coli H1443 *aroB*	0.16	0.04
E. coli H873 *aroB fepA*	0.02	0.04
E. coli H1594 *aroB fiu*	0.32	0.04
E. coli H2222 *aroB cir*	0.16	0.04
E. coli H1728 *aroB cir fiu*	0.32	0.02
E. coli H1875 *aroB fepA cir*	0.16	0.04
E. coli H1877 *aroB fepA fiu*	1.25	0.32
E. coli H1876 *aroB fepA cir fiu*	>10	8.50

Minimal inhibitory concentration (MIC) is defined as the highest concentration that gives a total inhibition of growth in liquid medium. Peptide concentrations were assayed in the range of 0.02–10 μM.

Conclusions

The use of high affinity outer membrane receptors involved in iron uptake for cell entry is a strategy not only MccE492 has adopted, but also other microcins as shown for MccJ25 (Salomón & Farías 1993; Destoumieux-Garzón *et al.* 2005), colicins (for review see Braun *et al.* 2002; Lazzaroni *et al.* 2002), antibiotics including albomycin (Ferguson *et al.* 2000), and tailed phages such as phage T5 (Heller & Schwarz 1985; Boulanger *et al.* 1996).

The microcin E492 model differs from the parasitism mechanisms mentioned here in that the peptide has adopted a siderophore as a post-translational modification. According to our

hypothesis, such a modification is likely to provide the uptake system with a higher efficiency (see above). From our current knowledge on microcin gene clusters, two other polypeptides, which remain to be isolated, could naturally adopt a siderophore-type posttranslational modification. Indeed, as discussed by Patzer *et al*. (2003), the sequences of MccM and H47 (deduced from their nucleic acid sequence) are highly similar to MccE492 C-terminal sequence, which carries the posttranslational modification in MccE492m. In addition, all three peptides display closely related genetic systems. The hypothesis that MccH47 could be synthesized as a siderophore-peptide is also supported by recent data showing that inactivation of genes involved in enterobactin biosynthesis led to a dramatic decrease in the antibacterial activity of MccH47-producing strains (Azpiroz & Laviña 2004). Interestingly, a similar strategy applied to a MccE492-producing strain resulted in the production of unmodified MccE492 only (Thomas *et al*. 2004). This strongly suggests that similar to MccE492, MccH47 could increase its specific activity by acquiring a modification deriving from catecholate siderophores. Finally, the finding that a *fepA cir fiu* triple mutant is resistant to the bacterial secretions of MccM and MccH47 producing strains (Patzer *et al*. 2003) is consistent with the hypothesis of a family of siderophore-peptides that use iron-uptake systems for

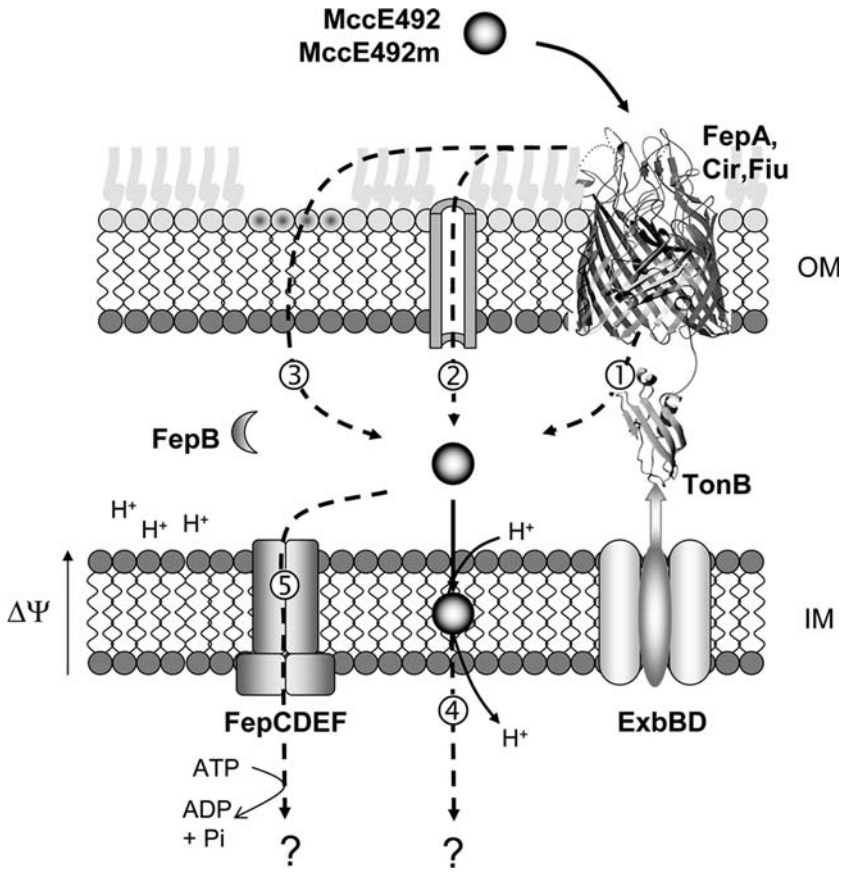


Figure 7. Schematic model of MccE492/MccE492m recognition and translocation. After recognition by the FepA, Cir and Fiu cathecolate siderophore receptors, the microcins are translocated across the outer membrane (OM) through an energy and TonB-dependent mechanism. To circumvent the OM permeability barrier (mainly due to lipopolysaccharides), translocation may occur via passage through the receptor β-barrel (1), a porin (2), or a region with different lipid composition such as a 'Membrane Island' (3). Once in the periplasmic space, MccE492/MccE492m insert into the cytoplasmic membrane (IM) inducing proton leakage and subsequent drop of the IM potential ($\Delta\psi$). At this stage, it is not known whether the microcins reach the cytoplasm. Passage across the IM may either be self-promoted (4) or involve a membrane transporter (5). Structures for FepA and TonB C-terminal domain are available under the 1FEP and 1XX3 PDB references, respectively.

improved recognition and entry into target cells. Until now, difficulty to obtain pure peptide preparations has been the limiting step for studying MccH47 and MccM structure and mechanism of action. No doubt that future studies will help elucidating the question on whether siderophore-peptides form a broader family of natural antimicrobial peptides.

Following a recognition step involving the high affinity iron-siderophore receptors FepA, Cir and Fiu at the outer membrane, MccE492/MccE492m are translocated across the outer membrane (OM) through an energy-dependent mechanism that requires the TonB machinery (Destoumieux-Garzón *et al*. 2003) (Figure 7). Experiments on spherosplasts, which bypass the recognition step, have shown that the recognition and/or translocation step across the outer membrane is a limiting factor for the MccE492 activity (Strahsburger *et al*. 2005). At this time, the mechanism by which translocation happens remains a puzzling question. The need for both energy and TonB was also reported for other microcins and group B colicins (for review see Braun *et al*. 2002), but the translocation processes remain unsolved. As discussed for colicins, we do not know whether MccE492/MccE492m translocation occurs (i) by passage through the receptor β-barrel after unfolding of the microcin, or (ii) away from the initial microcin binding-site, either by passage through a porin channel or by insertion into membrane regions displaying a weaker permeability barrier as 'Membrane Islands' could be (Cao & Klebba 2002).

Our previous studies have shown that once inside the periplasm, MccE492 is able to insert into the cytoplasmic membrane and that membrane insertion can occur via both the N- and C-terminal ends (Destoumieux-Garzón *et al*. 2003). However, as discussed earlier, the membrane permeabilization process, which promotes inner membrane depolarization (Figure 7), cannot be considered by itself responsible for the microcin killing activity. Given that no clues are available as regards the localization of other cellular targets for the microcin, one could suggest that insertion into the cytoplasmic membrane is part of a translocation process giving access to a potential cytoplasmic target. Alternatively, such an hypothetical translocation may follow the iron-uptake pathway and involve membrane permease and ATP-binding cassette transporters (Andrews *et al*. 2003). Future studies dedicated to the identification of MccE492 cellular targets will be of great interest in order to better characterize the final steps of MccE492 translocation process, and fully understand the mechanism of antibacterial activity.

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