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The polypeptide core of Microcin E492 stably associates with the mannose permease and interferes with mannose metabolism

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Abstract

Microcin E492 (MccE492) is an antibacterial protein whose activity on target cells requires ManYZ, the inner membrane component of the mannose permease. We show here that MceA, the polypeptide core of MccE492, stably associates with ManYZ both in the presence and in the absence of MceB, the MccE492 immunity protein. The two known physiological activities of the mannose permease were assayed in cells co-expressing MceA and MceB. Under these conditions, growth on mannose as the sole carbon source is prevented; this was not observed in cells expressing only MceB. In contrast, susceptibility to bacteriophage λ infection was not affected.

Keywords: MceA; Bacteriocin; Immunity; Mannose; Bacteriophage λ

1. Introduction

Bacteriocins are proteinaceous antibiotics produced by bacteria. Microcin E492 (MccE492) is secreted by Klebsiella pneumoniae RYC492 and active against various Enterobacteriaceae (de Lorenzo, 1984; de Lorenzo et al., 1984; Thomas et al., 2004). It is composed of a polypeptide core (MceA; Swiss-Prot Q9Z4N4, aa 16-99) with a C-terminal covalent modification (Thomas et al., 2004). MccE492 is believed to form pores in the inner membrane, leading to lethal disruption of membrane potential and integrity (de Lorenzo and Pugsley, 1985; Destoumieux-Garzon et al., 2003). Bacteria producing MccE492 synthesize an immunity protein (MceB), which is also localized in the inner membrane (Lagos et al., 1999).

MccE492 bactericidal activity is strictly dependent on the inner membrane component of the mannose permease (Biéler et al., 2006). This permease is composed of two membrane proteins (ManY and ManZ) and one cytoplasmic protein (ManX) that are involved in the uptake of mannose and related hexoses (Elliott and Arber, 1978; Erni et al., 1987; Williams et al., 1986). The mannose permease is also required for bacteriophage λ DNA penetration across the inner membrane (Elliott and Arber, 1978; Erni et al., 1987).

The expression of MceA, the polypeptide core of MccE492, is toxic in itself. This activity is enhanced by targeting to the inner membrane, for instance, with a mutant PhoA signal sequence (PhoA73) (Biéler et al., 2006). Bacteria that express the immunity protein MceB or that do not synthesize ManYZ are insensitive to MccE492, supplied extracellularly, and to both MceA and PhoA73-MceA, which exert their effect from the cytoplasm (Biéler et al., 2006). It is not yet known whether MceB acts by preventing MceA interaction with the mannose permease or by interfering at a later stage with the formation of toxic pores. In the latter case, MceB could, with or without MceA, affect the known functions of the mannose permease.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strains AB3 (ΔmanXYZ::cat) and AB4 (manY::Tn10kan) are derivatives of DB503 (Biéler et al.,
pBAD33 and pBAD101 have been described (Bie勒 et al., 2006; Guzman et al., 1995). pAB11 expresses PhoA73-MceA fusion under the control of the arabinose P_{BAD} promoter (Bie勒 et al., 2006). pAB23 expresses only MceB under the control of the P_{BAD} promoter of pBAD33 and is derived from pAB6 (Bie勒 et al., 2006). pJFLPM expresses ManX, ManY and untagged ManZ under the control of P_{tac}; pJFP-H6M expresses ManY and hexahistidine (6H)-tagged ManZ (Esquinas-Rychen and Erni, 2001).

2.2. Membrane preparation

Cell fractionation was performed as described (Oliver and Beckwith, 1982), with the following modifications: cells were grown at 37°C to an A_{600} of 0.6 in 20 ml of LB and induced as indicated. Cells washed twice in 10 mM Tris pH 8 were resuspended in 2 ml of 20% sucrose, 30 mM Tris pH 8. After addition of 200 μl of lysozyme (0.5 mg/ml in 50 mM EDTA), cells were incubated for 15 min on ice. After addition of 40 μl of 1 M MgCl2, the spheroplasts were centrifuged for 10 min at 1500 × g at 4°C, resuspended in 2 ml of 10 mM Tris pH 8, 0.1 M NaCl, 2 mM MgCl2, 1 mM CaCl2, 8 U/ml DNase (Promega), frozen and thawed thrice, and finally centrifuged at 100,000 × g for 1 h at 4°C. Membrane pellets were resuspended in 100 μl of 50 mM Tris pH 8, 0.1 M NaCl.

2.3. Purification and detection of hexahistidine (6H)-tagged protein complexes

Membranes (20 μl) were solubilized in DDM buffer (20 mM Tris pH 8.1, 0.3 M NaCl, 10% glycerol, 1% n-dodecyl-β-D-maltoside (DDM), 2 mM MgCl2, 10 mM imidazole and 3.4 mg/ml E. coli polar lipids (Avanti Polar Lipids, Inc.)) or in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with 2 mM MgCl2 at 4°C for 1 h with agitation and centrifuged for 15 min at 13,000 × g at 4°C. Supernatants were incubated at 4°C for 30 min with 20 μl of Ni-NTA agarose (Qiagen) and washed thrice with 1 ml of DDM buffer containing 30 mM imidazole and 1.5 mg/ml E. coli lipids or RIPA buffer. Pellets were resuspended in 40 μl of SBX (80 mM Tris pH 6.8, 1% β-mercaptoethanol, 2% SDS, 0.01% bromophenol blue, 10% glycerol, 10 mM EDTA), incubated for 20 min at 37°C and eluates were loaded onto 16% SDS-polyacrylamide gels. MceA immunoblotting was performed as described (Bie勒 et al., 2006).

3. Results and discussion

3.1. MceA stably associates with ManYZ

A link between the activity of MceE492 and the presence of ManYZ had been established genetically (Bie勒 et al., 2006). Fig. 1 shows that PhoA73-MceA co-purified with 6H-tagged ManZ, while no PhoA73-MceA was recovered with cells expressing wild-type, untagged ManZ, from the pJFLPM plasmid. Control experiments showed that SecY, an inner membrane protein that only interacts transiently with ManY and ManZ during their insertion in the membrane, was not detectably associated with 6H-tagged or untagged ManZ (Fig. 1A). This association is remarkably stable, since it is not only resistant to mild detergent (DDM buffer, Fig. 1A), but also to stronger detergents (RIPA buffer, Fig. 1B). PhoA73-MceA and ManZ may associate either through a direct interaction or through an indirect one involving, for example, ManY. Indeed, ManY and ManZ form a stable complex in the inner membrane (B. Erni, personal communication). These biochemical results indicate that a stable physical interaction can be detected between the mannose permease and the polypeptide core of MccE492.

![Fig. 1. Binding of PhoA73-MceA to ManYZ in the absence or in the presence of MceB. Cultures of AB4 pJFLPM (ManYZ) or AB4 pJFP-H6M (ManY 6H-ManZ) harboring pAB11 (PhoA73-MceA) (+) or the pBAD101 vector (−), and pAB23 (MceB) (+) or the pBAD33 vector (−), were induced for 10 min with 0.2% arabinose and 0.5 mM IPTG and fractionated. (A) Membranes were analyzed by MceA immunoblotting either directly (top, 5 μl of membranes) or after solubilization in DDM buffer and purification of 6H-tagged protein complexes (bottom, 20 μl of membranes). The blot was reprobed with an anti-SecY antibody. (B) Membranes were solubilized in RIPA buffer.](image-url)
3.2. MceB does not prevent the association of MceA with ManYZ

The association of PhoA73-MceA with ManYZ is not prevented by the expression of the immunity protein MceB (Fig. 1). Thus, MceB does not exert its protective action by blocking PhoA73-MceA membrane insertion or by preventing PhoA73-MceA from interacting with ManYZ (Fig. 2, model 2a). Therefore, MceB is most likely to be active at a later stage, for instance, by interfering with the toxic structure composed of both MceA and ManYZ. Since MceB synthesis abolishes PhoA73-MceA toxicity, higher amounts of PhoA73-MceA are produced in cells expressing MceB.

Interestingly, lactococcin A, a membrane-permeabilizing bacteriocin from the Gram-positive bacterium Lactococcus lactis, is also dependent on the membrane components of the mannose permease for bactericidal activity (Diep et al., 2007). Lactococcin A forms a stable complex with a tagged version of its cognate immunity protein and the mannose permease, but the immunity protein does not bind to the mannose permease in the absence of bacteriocin. Similar results were obtained with several other bacteriocins, including Listeria-active ones, which do not share sequence homology with MceA or lactococcin A (Diep et al., 2007).

3.3. Co-expression of MceA and MceB interferes with mannose metabolism

Since MceA associates with ManYZ, MceA expression could affect the two other known functions of the mannose permease (Fig. 2, model 2b). We tested these possibilities by measuring the capacity of cells stably expressing PhoA73-MceA and MceB to use mannose as a carbon source (Man$^+$ or Man$^-$ phenotypes) and to be infected by bacteriophage $\lambda$ ($\lambda^S$ or $\lambda^R$ phenotypes). Strikingly, mannose metabolism is blocked when the expression of PhoA73-MceA and MceB is induced (Man$^-$ phenotype), as evidenced by strong cell growth inhibition on minimal-mannose medium (Fig. 3A), while growth is normal on minimal-glycerol medium (Fig. 3B). Similarly, no mannose fermentation was detected on McConkey indicator plates upon PhoA73-MceA and MceB co-expression (data not shown). In contrast, expression of MceB alone had no visible effect on mannose metabolism. Since expression of PhoA73-MceA without MceB is lethal, its effect on mannose metabolism cannot be assessed.

The inhibition of growth observed upon induction of MceA and MceB was quantified in liquid cultures. In minimal-mannose medium, the doubling time increased by more than 7-fold upon induction; in contrast, growth in minimal glucose medium was only slightly decreased upon induction (Fig. 3C). This probably reflects the fact that most of the glucose used under our culture conditions is imported by the PtsG system. The situation is strikingly different in Lactococcus cells expressing the immunity protein and exposed to extracellular lactococcin A, since growth in the presence of mannose was barely affected (Diep et al., 2007).

Finally, growth in liquid cultures led us to determine that growth inhibition provoked by induction of MceA and MceB is reversible, since growth immediately resumes upon addition of glucose to induced cells (Fig. 3D).

In contrast, sensitivity to bacteriophage $\lambda$vir infection, which makes clear plaques with sharp edges, is not significantly altered by PhoA73-MceA and MceB co-expression ($\lambda^S$ phenotype; data not shown). Thus, distinct portions of the mannose permease are likely to be involved in MceE492 sensitivity and bacteriophage DNA penetration (Fig. 2, model 2b). Since $\lambda$ infection requires much less ManYZ activity than growth on mannose, a difference in the kinetics of plaque formation may not be visible, but the plaques were neither small nor minute. Although ManYZ inhibition by MceA and MceB has a measurable effect on mannose
transport, it may not prevent the occasional penetration of a single bacteriophage DNA molecule.

Although it is not known whether bacteriophage DNA penetration and mannose uptake involve the same structural element, the diameter of a double-stranded DNA is significantly larger than that of mannose, a monosaccharide. Thus, the transfer of DNA across the inner membrane may not require the conformational changes necessary for mannose uptake. The stable expression of MceA and MceB offers new tools to decipher the multiple functions of the *E. coli* mannose transport system.

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References


