

## Inhibition of *Salmonella enterica* serovars by microcin J25

Paula A. Vincent, Mónica A. Delgado, Ricardo N. Farías, Raúl A. Salomón \*

Departamento de Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Tucumán), and Instituto de Química Biológica "Dr. Bernabé Bloj", Chacabuco 461, 4000 San Miguel de Tucumán, Tucumán, Argentina

Received 16 April 2004; received in revised form 14 May 2004; accepted 18 May 2004

First published online 28 May 2004

### Abstract

*Escherichia coli* microcin J25 (MccJ25) is a 2107-Da peptide antibiotic whose uptake into *E. coli* is mediated by the outer-membrane receptor FhuA and the inner membrane proteins TonB, ExbB, ExbD, and SbmA. A survey of the sensitivity of several *Salmonella enterica* serovars showed that the antibiotic was highly active against some serovars, while *S. Typhimurium*, *S. Derby*, and some *S. Enteritidis* strains were completely resistant. Resistant strains became hypersensitive to MccJ25 when given the *fhuA* gene of *E. coli*, indicating that insensitivity is due to the inability of the FhuA protein to mediate penetration of MccJ25. Whereas in *E. coli* MccJ25 targets RNA polymerase, in *S. Typhimurium* it inhibits not only RNA synthesis but also cell respiration. Fluorescence viability staining showed that *S. Typhimurium* cells exposed to MccJ25 remain viable but are unable to form colonies. © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** Microcin J25; *Salmonella*; FhuA; Mode of action

### 1. Introduction

Microcin J25 (MccJ25) is a plasmid-encoded, 2,107-Da peptide antibiotic of 21 unmodified amino acids, excreted to the culture medium by an enteric *Escherichia coli* strain [1,2]. MccJ25 uptake into *E. coli* is dependent on the outer-membrane receptor FhuA [3] and the inner membrane proteins TonB, ExbB, ExbD, and SbmA [4]. We are currently interested in the action of MccJ25 on salmonellae, which are facultative intracellular pathogens responsible for a variety of diseases in a wide variety of animal species. In humans, they cause illnesses ranging from localized gastroenteritis (food poisoning) to septicemia and typhoid fever.

Our long-term objective is the development of MccJ25 as a useful antibiotic. To this end, several issues must be clarified, such as its in vivo efficacy. To this end,

we envisage to use the well-established murine model of infection with *S. Typhimurium*. However, this is hampered by the fact that serovar *Typhimurium* behaves as completely resistant to MccJ25. In the present study, we demonstrate that the intrinsic resistance of *S. Typhimurium* and other *Salmonella* serovars is due to the inability of the FhuA protein to mediate the penetration of MccJ25, since introduction of the *E. coli fhuA* allele cloned in a multicopy plasmid into these bacteria rendered them hypersensitive to the antibiotic. In fact, exposure of the transformed *S. Typhimurium* to MccJ25 caused a precipitous drop in colony counts. Of note, fluorescence viability staining suggested that at least during the first 2 h of exposure to the antibiotic the cells remain alive but are unable to form colonies. We also show that in *S. Typhimurium* MccJ25 targets not only RNA polymerase (as in *E. coli*) but also cell respiration, and propose that the hypersusceptibility of *Salmonella* species to the antibiotic could result from the additive effect of these two modes of action.

\* Corresponding author. Tel./fax: +54-381-4248921.

E-mail address: salomon@unt.edu.ar (R.A. Salomón).

## 2. Materials and methods

### 2.1. Bacterial strains and plasmid

Except for *S. Typhimurium* SL3770 and SL1277, which are derivatives of strain LT2, all *Salmonella* strains used here were clinical isolates of independent origin. Strains MC4100 [*araD139*  $\Delta$ (*argF-lac*)205  $\lambda^-$  *flbB5301 rpsL150 relA1 deoC1 ptsF25 rbsR*] and KL723 [F'104 *thr-1 leuB6*  $\Delta$ (*gpt-proA*)62 *hisG4 argE3*] are derivatives of *E. coli* K-12. Plasmid pGC01 is a pBR322 derivative carrying *E. coli fhuA* cloned [5].

### 2.2. Media and growth conditions

Luria broth (LB) and M9 minimal salts were purchased from Sigma Chemical Co. Minimal medium was supplemented with glucose (0.2%), MgSO<sub>4</sub> (1  $\mu$ g/ml), and vitamin B<sub>1</sub> (1  $\mu$ g/ml). Agar was added to a 1.5% concentration to prepare solid media. Cells containing plasmid pGC01 were grown in the presence of ampicillin at 50  $\mu$ g/ml. All cultures, solid and liquid, were grown at 37 °C.

### 2.3. DNA manipulations

Plasmid DNA was isolated by the alkali lysis procedure [6]. Electroporation of *Salmonella* strains was carried out with a MicroPulser apparatus (BioRad) as recommended by the supplier.

### 2.4. MccJ25 purification and susceptibility determinations

MccJ25 was purified as described previously [1]. For a comparison of the sensitivities of different strains, we used a spot-on-lawn test [2] or determined the MIC of microcin for each strain [2].

### 2.5. Viability assessment by fluorescence staining

A culture of *S. Typhimurium* SL3770 (pGC01) growing exponentially (DO<sub>600</sub> = 0.3) in LB was divided into two parts. One of them received microcin at 0.5  $\mu$ M, while the other was run as a control. At 2 h, 500  $\mu$ l samples from both cultures were transferred to Eppendorf tubes and stained with 5  $\mu$ M SYTOX Green stain (Molecular Probes Inc., Eugene, OR) and the mixture was incubated for 2 h in a 30 °C water bath. Stained bacteria were observed with a Nikon Fluophot epifluorescence microscope. Viability was determined by counting the number of green fluorescent dead cells for a given field of view. A minimum of three fields, with more than 100 cells per field, was counted in each experiment.

### 2.6. Effect of MccJ25 on the in vivo incorporation of labeled uridine on RNA

The effect of MccJ25 on in vivo transcription by *S. Typhimurium* SL3770 (pGC01) was performed as described [7].

## 3. Results

### 3.1. MccJ25 sensitivity of various *Salmonella enterica* serovars

The sensitivity to MccJ25 of *S. Typhimurium* SL3770, and independent clinical isolates of *S. Typhimurium* (11 isolates), *S. Enteritidis* (6 isolates), *S. Newport*, *S. Typhi*, *S. Paratyphi B*, *S. Heidelberg* and *S. Derby* (one strain each) was quantitated using the serial double dilution assay (Table 1). Serovars Heidelberg, Newport, Enteritidis, and Paratyphi B were 30–130 fold more sensitive to MccJ25 than the widely used laboratory strain of *E. coli* K-12 MC4100. In a liquid-based antibacterial assay in M9-glucose, the MIC of MccJ25 for *S. Paratyphi B* was as low as 4 nM, 30-fold lower than that for MC4100. It is of note that three strains of *S. Enteritidis* (out of six tested) were resistant to MccJ25. *S. Typhi* was nearly resistant [even undiluted MccJ25 (500  $\mu$ M) gave a very turbid halo of inhibition], whereas *S. Derby* and all strains of *S. Typhimurium* tested behaved as completely resistant to MccJ25 (Table 1).

### 3.2. *S. Typhimurium* becomes fully sensitive to MccJ25 when provided with *E. coli FhuA*

Killmann et al. [8] demonstrated that an *fhuA* mutant of *E. coli* expressing wild-type FhuA of *S. Typhimurium* is resistant to MccJ25, whereas FhuA of *S. Paratyphi B*

Table 1  
Comparison of the sensitivities of *E. coli* MC4100 and different serovars of *S. enterica* to MccJ25

Strain	Sensitivity
<i>E. coli</i> MC4100	64
<i>S. Typhimurium</i> <sup>a</sup>	–
<i>S. Typhi</i>	(1)
<i>S. Newport</i>	4096
<i>S. Enteritidis</i>	4096
<i>S. Derby</i>	–
<i>S. Heidelberg</i>	2048
<i>S. Paratyphi B</i>	8192

Doubling dilutions of MccJ25 were applied onto plates of M9-glucose supplemented with 0.1% tryptone, which were then seeded with the strain to be tested. The results are given as the reciprocal of the last dilution which produced a clear spot (in parentheses, turbid zone). –, no inhibition.

<sup>a</sup> *S. Typhimurium* SL3770 and 11 clinical strains were tested.

renders these cells sensitive to the antibiotic. This suggests that the insensitivity of *S. Typhimurium* to MccJ25 would only be due to the inability of its FhuA protein to bind or translocate MccJ25 across the outer membrane. However, the possibility existed that *S. Typhimurium* might also be altered in a step after the receptor or at the site of action; if so, MccJ25 resistance would not be overcome by simply providing *E. coli* FhuA. To clarify this issue, the F' factor F'104 containing *E. coli fhuA* [9] was transferred from *E. coli* KL723 to a rough *S. Typhimurium* strain, SL1277, by conjugation, with selection on defined medium without proline. The absence of arginine and histidine was used to prevent growth of the donor strain. Only a few colonies were obtained, and all of them had become sensitive to MccJ25, suggesting that resistance of *S. Typhimurium* to MccJ25 is due to a deficiency in *fhuA* function. For unknown reasons, *S. Typhimurium* transconjugants carrying F'104 were unstable and gave rise to slow growing, small colonies that easily segregated larger colonies resistant to MccJ25. This unstable phenotype could complicate interpretation of future experiments. In addition, conferral of sensitivity by transformation with F'104 does not confirm that the insensitivity of *S. Typhimurium* is due to a deficiency of translocation, since the F' carries about 300 genes in addition to *fhuA*. It just suggests that it could be. For these reasons, we decided to transform into *S. Typhimurium* the plasmid pGC01 [5], which carries the *E. coli* wild-type *fhuA* gene. This plasmid was electroporated into *S. Typhimurium* SL3770, and transformants were selected in LB with ampicillin. SL3770 (pGC01) transformants became hypersusceptible to MccJ25. One of these transformants was approximately 130-fold more sensitive to MccJ25 than *E. coli* strain MC4100 in a spot-on-lawn test. This extreme susceptibility was also reflected in the MICs of MccJ25 for MC4100 and SL3770 (pGC01) in a liquid-based assay (100 and <0.2 nM, respectively). Importantly, the plasmid was stably maintained in its new host.

### 3.3. Heterogeneity of *Salmonella microcin* sensitivity

As described above, different serovars of *Salmonella enterica* differ widely in their sensitivities to MccJ25. On the basis of the preceding results, we think a likely explanation is an heterogeneity in the type of FhuA protein. To test this possibility, we electroporated pGC01 into MccJ25-resistant *S. Derby* and two MccJ25-resistant strains of *S. Enteritidis*. As with *S. Typhimurium*, the transformants became hypersensitive to microcin. Thus, not only different *Salmonella* serovars but even different strains of a defined serovar may be heterogeneous with respect to the type of *fhuA* gene.

### 3.4. Growth and survival kinetics of *S. Typhimurium* transformed with *fhuA*-carrying pGC01

We measured the effect of MccJ25 on growth and viability of SL3770 (pGC01). A culture was grown in LB to early exponential phase ( $OD_{600} = 0.3$ ) and split into two portions. One of them received microcin to a final concentration of 0.5  $\mu\text{M}$ , while the other was run as a control. At intervals, samples were removed, diluted, and plated in LB. As can be seen in Fig. 1(a), MccJ25 addition caused an almost immediate leveling off of turbidity, and the absorbance remained unchanged until the end of the experiment, indicating that the peptide exerts its antibacterial action without causing cell lysis. After only 5 min of mixing the cells with MccJ25 colony counts had dropped from  $10^8$  to about  $10^6$  CFU/ml (Fig. 1(b)). This initial phase of rapid decline in viable counts was followed by a more gradual decrease, and after 60 min of exposure to microcin viability had decreased two orders of magnitude more.

### 3.5. Viability assessment by staining of bacterial suspensions with SYTOX Green stain

The rapid decline in viable-cell counts suggested an immediate effect of microcin on cells. However, we could

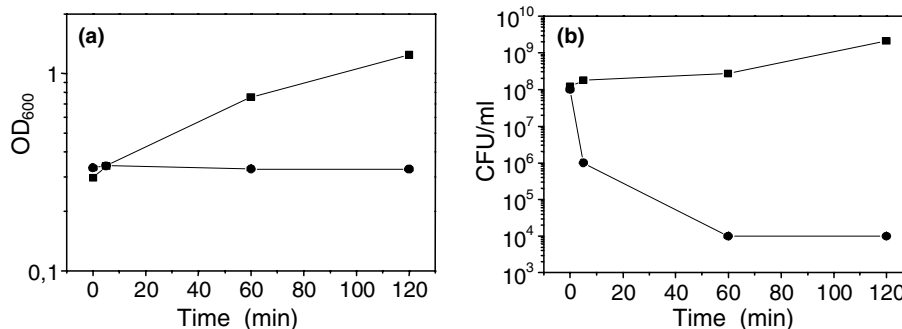


Fig. 1. Effect of MccJ25 on growth and survival of *S. Typhimurium* SL3770 (pGC01). A culture was grown overnight in LB and then diluted 100-fold in the same medium. When it reached an  $OD_{600}$  of about 0.3, it was split into halves, one of which was challenged with 0.25  $\mu\text{M}$  MccJ25 (●), and the other was kept as a control (■). Microcin was added at time zero in the graphs. Growth ( $OD_{600}$ ) (a) and the number of viable cells (b) were determined at the indicated times.

not rule out a slower, continued action of MccJ25 (rather than an immediate effect) as it remained associated with the cells during incubation on the agar plate. To distinguish between these two possibilities, we determined the immediate effect of MccJ25 on cell viability by using fluorescence viability staining with SYTOX Green nucleic acid stain. This is a high-affinity probe that easily penetrates Gram-negative bacteria with compromised plasma membranes, yet it is completely excluded from live cells [10]. Samples from the cultures were stained as indicated in Section 2 and examined microscopically to determine the ratio of viable to dead cells. Viability of SL3770 (pGC01) exposed to MccJ25, as determined by fluorescence staining, was much higher than that indicated by plate counts. Only a very small fraction of the organisms (less than 0.5%) were bright green fluorescent (dead) (Fig. 2). Viability remained as high as 98% even after 2 h exposure to microcin. Furthermore, microscopic examination of microcin-treated cells did not indicate any obvious disruption of cell integrity. The discrepancy seen between the plate counts and fluorescence staining viability assays may be explained by assuming that most of cells remain alive but are unable to grow and form colonies.

### 3.6. Effect of MccJ25 on oxygen consumption and transcription in *S. Typhimurium* (pGC01)

Previously, we have reported that *E. coli* RNA polymerase is the target of MccJ25 and that the *in vivo* and *in vitro* transcription activity of the enzyme is inhibited by the antibiotic [7,11]. On the other hand, work from our laboratory using a strain of *S. Newport* as a test organism showed that MccJ25 dissipates the membrane potential and inhibits respiration [12] but, remarkably, these effects were not seen with *E. coli*. In this study, we wanted to determine the physiological and biochemical effects of MccJ25 on a different bacterial species, *S. Typhimurium*.

First, the effect of MccJ25 (0.5  $\mu\text{M}$ ) on respiration of logarithmic-phase LB cultures ( $\text{OD}_{600}$ , 0.3) of aerobi-

cally grown *S. Typhimurium* (pGC01) was measured in a 3-ml incubation vessel fitted with a Clark-type oxygen electrode. The antibiotic caused a marked inhibition of oxygen consumption, and this effect was rather rapid since it became already evident at 5 min after the addition of MccJ25 to the electrode vessel (Fig. 3). We wished to know whether MccJ25 targets *S. Typhimurium* RNA polymerase as well. Amino acids that, when mutated, cause resistance to MccJ25 are part of evolutionary conserved segments F, G, and G' of the  $\beta'$  subunit from *E. coli* RNA polymerase [11]. Sequence alignment with the corresponding regions of  $\beta'$  from *S. Typhimurium* (Accession No NP\_463023) revealed a perfect conservation, suggesting that MccJ25 could also target RNA polymerase in *S. Typhimurium*. Whole-cell RNA labeling studies for *S. Typhimurium* SL3770 (pGC01) confirmed this presumption, since within 5 min of addition of MccJ25 (0.05  $\mu\text{M}$ ) incorporation of radiolabeled precursor into RNA ceased. Note that within

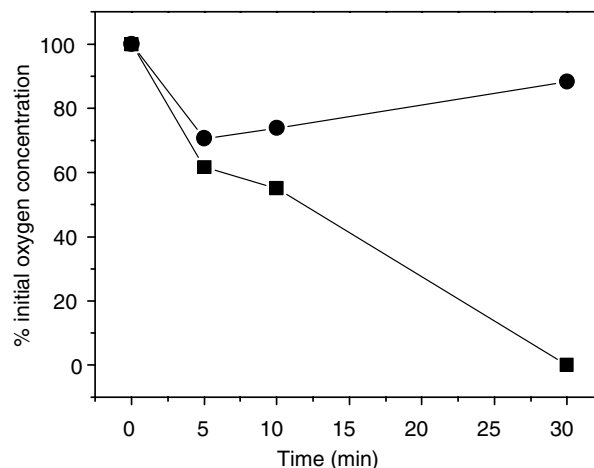


Fig. 3. Effect of MccJ25 on oxygen consumption of *S. Typhimurium* (pGC01). Oxygen consumption in the presence (●) and in the absence (■) of 0.5  $\mu\text{M}$  MccJ25 is shown as percentage of the initial oxygen concentration in air-saturated LB culture medium. MccJ25 was added at time zero. The results shown are representative of several very similar, independent experiments.

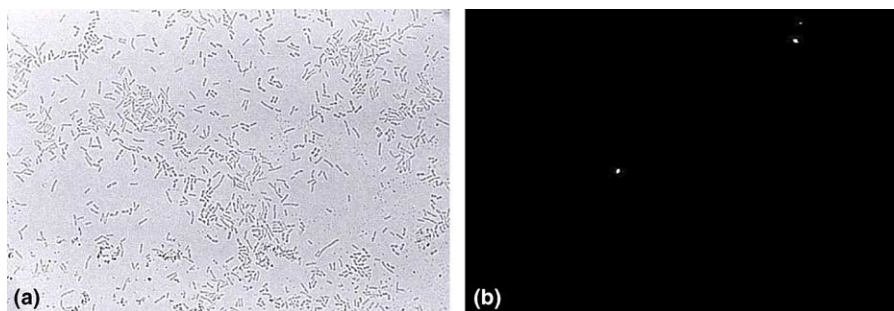


Fig. 2. Fluorescence viability staining with SYTOX Green nucleic acid stain. Panels: (a) representative micrograph of exponentially growing cells of strain SL3770 (pGC01) treated with MccJ25 and SYTOX as indicated in Section 2; (b) the same field observed by epifluorescence (excitation, 490 nm). Only two cells were stained with bright green fluorescence.

the time frame of this experiment (30 min) the cells remained viable, as indicated by fluorescence staining (see the preceding paragraph). Taken together, these results indicated a dual mode of action of MccJ25 on *Salmonella*.

#### 4. Discussion

A relevant aspect of this work is the finding that *Salmonella* serovars that are naturally resistant to MccJ25 become sensitive when provided with *E. coli* FhuA. Therefore, of the genes needed for MccJ25 action, *S. Typhimurium*, *S. Derby* and some strains of *S. Enteritidis* are deficient only in the *fhuA* function. We also show that in *S. Typhimurium* MccJ25 inhibits not only RNA polymerase but also cell respiration. At present, we do not know which one is the primary mode of action in this bacterium. In any case, we want to suggest that the extreme susceptibility of *Salmonella* serovars to MccJ25, as compared with *E. coli* (where RNA polymerase seems to be the unique target), could result from an additive interaction of both mechanisms. It is of note that our experiments with SYTOX Green stain indicate that important damage to the cell membrane permeability does not occur during treatment with the antibiotic. So, we believe that inhibition of respiration does not result from disruption of the membrane integrity, but rather of a direct interaction of MccJ25 with one or more components of the respiratory chain.

Another interesting finding of the present study is that MccJ25 seems to have a long lasting effect on *Salmonella*. In fact, cells exposed to the antibiotic remain viable (as judged from fluorescence viability staining) but are unable to grow and form colonies. Thus, MccJ25 behaves as a bacteriostatic agent or, at least, it exhibits a long bacteriostatic lag before a cidal action. Remarkably, microcin-induced bacteriostasis is different from that caused by conventional antibiotics in that cells do not recover after removal of the external antibiotic. In fact, dilutions before plating for the viability assays are great enough to eliminate further action of microcin. The sustained inhibition of growth may be due to an extremely tight association of microcin with the cells. In contrast to conventional antibiotics, which can freely diffuse into or out of the cells, microcin would remain trapped in the cytoplasm once internalized, since it might not permeate through the inner membrane and its molecular mass (2107 Da) and high hydrophobicity would prevent its passage through porins, whose molecular mass cutoff is 600 Da. By contrast, inhibition of *E. coli* cells is relieved once microcin is removed from

the medium [2]. The molecular basis of this differential behavior is currently under study.

#### Acknowledgements

We thank J. Coulton, Mary Berlyn (at the *E. coli* Genetic Stock Center), Instituto Malbrán (Buenos Aires) and Instituto de Microbiología of the Universidad Nacional de Tucumán for the gift of bacterial strains. This work was funded by ANPCYT (grant PICT 7231) and CIUNT (grant 26/D235). P.A.V. and M.A.D. were recipients of postdoctoral fellowships from CONICET. R.A.S. and R.N.F. are career investigators of CONICET.

#### References

- [1] Blond, A., Péduzzi, J., Goulard, C., Chiuchiolo, M.J., Barthélémy, M., Prigent, Y., Salomón, R.A., Fariás, R.N., Moreno, F. and Rebuffat, S. (1999) The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*. *Eur. J. Biochem.* 259, 747–755.
- [2] Salomón, R.A. and Fariás, R.N. (1992) Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. *J. Bacteriol.* 174, 7428–7435.
- [3] Salomón, R.A. and Fariás, R.N. (1993) The FhuA protein is involved in microcin 25 uptake. *J. Bacteriol.* 175, 7741–7742.
- [4] Salomón, R.A. and Fariás, R.N. (1995) The peptide antibiotic microcin 25 is imported through the TonB pathway and the SbmA protein. *J. Bacteriol.* 177, 3323–3325.
- [5] Carmel, G., Hellstern, D., Henning, D. and Coulton, J.W. (1990) Insertion mutagenesis of the gene encoding the ferrichrome-iron receptor of *Escherichia coli* K-12. *J. Bacteriol.* 172, 1861–1869.
- [6] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [7] Delgado, M.A., Rintoul, M.R., Fariás, R.N. and Salomón, R.A. (2001) *Escherichia coli* RNA polymerase is the target of the cyclopeptide antibiotic microcin J25. *J. Bacteriol.* 183, 4543–4550.
- [8] Killman, H., Braun, M., Herrmann, C. and Braun, V. (2001) FhuA barrel-cork hybrids are active transporters and receptors. *J. Bacteriol.* 183, 3476–3487.
- [9] Low, K.B. (1972) *Escherichia coli* K-12 F-prime factors, old and new. *Bacteriol. Rev.* 36, 587–607.
- [10] Roth, B.L., Poot, M., Yue, S.T. and Millard, P. (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX Green nucleic acid stain. *Appl. Environ. Microbiol.* 63, 2421–2431.
- [11] Yuzenkova, J., Delgado, M., Nechaev, S., Savalia, D., Epshtein, V., Artsimovitch, I., Mooney, R.A., Landick, R., Fariás, R.N., Salomon, R. and Severinov, K. (2002) Mutations of bacterial RNA polymerase leading to resistance to microcin J25. *J. Biol. Chem.* 277, 50867–50875.
- [12] Rintoul, M.R., Fernández de Arcuri, B., Salomón, R.A., Fariás, R.N. and Morero, R.D. (2001) The antibacterial action of microcin J25: evidence for disruption of cytoplasmic membrane energization in *Salmonella newport*. *FEMS Microbiol. Lett.* 204, 265–270.