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Sensitization of Microcin J25-Resistant Strains by a Membrane-Permeabilizing Peptide[▽]

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Microcin J25 (MccJ25) is a plasmid-encoded, 21-amino-acid, antibacterial peptide produced by *Escherichia coli*. MccJ25 inhibits RNA polymerase and the membrane respiratory chain. MccJ25 uptake into *E. coli*-sensitive strains is mediated by the outer membrane receptor FhuA and the inner membrane proteins TonB, ExbB, ExbD, and SbmA. This peptide is active on some *E. coli*, *Salmonella*, and *Shigella* species strains, while other Gram-negative bacteria, such as clinical isolates of *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Moraxella catarrhalis*, and *Salmonella enterica* serovar Typhimurium, are completely resistant. In the present work, we demonstrated that the membrane-permeabilizing peptide $(KFF)_3K$ made some resistant strains sensitive to MccJ25, among them *S.* Typhimurium, where the antibiotic inhibits *in vitro* cell growth and bacterial replication within macrophages. The results demonstrate that the membrane permeabilization induced by $(KFF)_3K$ allows MccJ25 penetration in an FhuA and SbmA-independent manner and suggest that the combination of both peptides could be considered as a therapeutic agent against pathogenic *Salmonella* strains.

The antibiotic peptide microcin J25 (MccJ25), produced by an *Escherichia coli* strain, is ribosomally synthesized and consists of 21 amino acid residues (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) (4, 12). MccJ25 is a lasso peptide (1, 10, 17), contains a lactam linkage between the α-amino group of Gly^1 and the γ-carboxyl of Glu^8, forming an 8-residue ring (Gly^1 to Glu^8), which is termed a lariat ring. The "tail" (Tyr^9 to Gly^{21}) passes through the ring, with Phe^{19} and Tyr^{20} straddling each side of the tail, sterically trapping the tail within the ring. MccJ25 amino acids F^{10} to P^{16} form a β-hairpin structure comprising two β-strands (F^{10}-V^{11} and T^{15}-P^{16}) and a β-turn (V^{11} to G^{14}).

The uptake of MccJ25 into the *E. coli* periplasmic space depends on the outer membrane receptor FhuA and the inner membrane proteins TonB, ExbD, and ExbB (11, 13). Additionally, the inner membrane protein SbmA transports MccJ25 from the periplasmic to the cytoplasmic space (13). Once inside the sensitive cell, the peptide is able to inhibit *E. coli* RNA polymerase (RNAP) and membrane respiratory chain, which represent the MccJ25 targets (2, 5, 7, 18). Several *Salmonella enterica* serovars showed high sensitivity against MccJ25, while others, like *Salmonella enterica* serovar Typhimurium, *S.* enterica serovar Derby, and some *S. enterica* serovar Enteritidis strains were completely resistant (16). Since introduction of the *E. coli fhuA* allele cloned in a multicopy plasmid into these bacteria rendered them hypersensitive to the antibiotic, we concluded that this intrinsic resistance is due to the inability of the FhuA receptor protein to mediate the penetration of MccJ25. In fact, MccJ25 was able to inhibit both intracellular targets in the transformed strains (16).

The polianionic lipopolysaccharide (LPS) component of the outer membrane is stabilized by divalent cation bridges (15). It was suggested that many cationic peptides are able to bind to LPS and disrupt these bridges, resulting in an increased bacterial membrane permeabilization. Vaara and Porro (15) characterized a series of synthetic peptides that were able to sensitize Gram-negative bacteria to hydrophobic and amphipathic antibiotics. One of them, KFFKFFKFFK [$(KFF)_3K$], a peptide rich in cationic lysine and hydrophobic phenylalanine residues, showed a potent effect on outer membrane disorganization and weak damage to the cytoplasmic membrane (15).

In this work, we have shown that the $(KFF)_3K$ peptide allows MccJ25 uptake independently of the FhuA and SbmA receptors, turning *in vitro* microcin-resistant strains into susceptible ones. Moreover, we have demonstrated that $(KFF)_3K$ was able to exert the same inhibitory effect *in vivo* on *S.* Typhimurium replicating within eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) or minimal medium M9 (14). When necessary, kanamycin was added at a final concentration of 50 μg/ml.

All clinical isolates used (Table 1) are deposited in our collection at the Department of Biología de la Nutrición, INSIBIO, UNT-CONICET, Tucumán, Argentina.

MccJ25 sensitivity assay. Sensitivity to MccJ25 was measured by determination of the minimal concentration able to inhibit the bacterial growth (MIC) or by a viable recount in plates (CFU). The MICs were measured by the 2-fold serial dilution assay. Exponentially growing cultures of the tested strains (optical density at 600 nm [OD_{600}] = 0.4 to 0.5) were suitably diluted in M9-Casamino Acids medium, and 10^4 cells were added to tubes containing 0.25 ml of MccJ25

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TABLE 1. Bacterial strains used in this study

Strain	Description	Reference or source[a]
E. coli		
DH5α	*endA1 hsdR17* (rK^- mK^+) *supE44 thi-1 recA1 gyrA relA1*(*lacIZYA-argF*) *U169deoR* [ϕ80*d lacZ*M15]	BRL
MC4100	F^- *araD139* Δ(*argF-lac*)*205* λ^- *rpsL150 flbB5301 relA1 deoC1 pstF25*	CGSC
AB259	HfrH *supQ*80 λ^- *relA1 spoT1 thi-1*	CGSC
SBG231	AB259 spontaneous $MccJ25^r$ mutant[b]	5
SG303 *fhuA*	MC4100 *aroB*	9
EZE100	MC4100 *sbmA*::Tn*5*	6
S. enterica serovar Typhimurium		
14028s	Wild type	8
SL3770	Wild type	SGSC
Serratia marcescens	Clinical isolates	UNT
Citrobacter freundii	Clinical isolates	UNT
Enterobacter cloacae	Clinical isolates	UNT
Klebsiella pneumoniae	Clinical isolates	UNT
Pseudomonas aeruginosa	Clinical isolates	UNT
Acinetobacter baumannii	Clinical isolates	MALBRAN
Moraxella catarrhalis	Clinical isolates	MALBRAN

[a] BRL, Bethesda Research Laboratory; CGSC, *E. coli* Genetic Stock Center; SGSC, *Salmonella* Genetic Stock Center; UNT, Universidad Nacional de Tucumán; MALBRAN, Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) Dr. Carlos Malbrán.
[b] $MccJ25^r$, microcin J25 resistant.

(470 μM) in 2-fold serial dilutions or the same MccJ25 dilutions and $(KFF)_3K$ (30 μg ml^{-1}). A control was carried out by incubating 10^4 cells with 30 μg ml^{-1} $(KFF)_3K$. Turbidity was read after 24 h of incubation at 37°C. The *S.* Typhimurium viable recount was done at different time points of bacterial growth in LB supplemented with either MccJ25 (117.5 μM), $(KFF)_3K$ (30 μg ml^{-1}), or MccJ25/$(KFF)_3K$ (117.5 μM/30 μg ml^{-1}) or in LB medium as a control. At indicated times, the CFU number for survival of each treatment was determined by subsequent plating on LB agar.

***In vivo* RNAP inhibition assay.** The *in vivo* transcription assay was carried out as described by Delgado et al. (7). Briefly, the wild-type *S.* Typhimurium 14028s strain was grown in M9-glucose (20 ml) to early exponential phase (OD_{600} = 0.2 to 0.3), and the culture was split into four 5-ml portions. They were supplemented with MccJ25 (117.5 μM), $(KFF)_3K$ (30 μg ml^{-1}), or MccJ25/$(KFF)_3K$ mix (117.5 μM/30 μg ml^{-1}), while the last one, containing no peptides, served as a control. At the same time, 15 mg of uridine per ml and 0.1 mCi of [^{3}H]uridine were added to the four cultures. At indicated times, 0.5 ml was removed from the flasks, mixed with 1.5 ml of 5% cold trichloroacetic acid (TCA), and chilled on ice for 1 h. Then, each sample was filtered through a Millipore HAWP02500 membrane and washed with 10 ml of cold TCA. The radioactivity retained on the dried membrane was estimated in a Beckman LS-1801 liquid scintillation counter.

Oxygen consumption inhibition. The rate of oxygen consumption was determined as described previously (3). The wild-type *S.* Typhimurium 14028s strain growing at exponential phase (OD_{600} = 0.4 to 0.5) in M9-Casamino Acids medium was incubated at 37°C with MccJ25 (117.5 μM), $(KFF)_3K$ (30 μg ml^{-1}), or MccJ25-$(KFF)_3K$ (117.5 μM and 30 μg ml^{-1}). A control was done without any added peptide. At different times, aliquots of 2 ml were removed and the average respiration rate for 5 min was polarographically measured with a Gilson Clark-type electrode oxygraph.

Bacterial replication within eukaryotic cells. RAW 264.7 macrophages were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS). Macrophages were seeded in 24-well plates at a density of 5×10^5 and grown at 37°C with 5% CO_2. Bacteria used to infect were grown in LB, washed with sterile phosphate-buffered saline (PBS), suspended in DMEM medium, and added to each well at a macrophage-to-bacterium ratio of 1:10. Bacterial infection was allowed to proceed for 20 min. Infected macrophages were washed three times with sterile PBS and incubated in fresh medium containing 100 mg ml^{-1} gentamicin. At this time point, MccJ25 (117.5 μM), $(KFF)_3K$ (30 μg ml^{-1}), or MccJ25-$(KFF)_3K$ mix (117.5 μM/30 μg ml^{-1}) were also added. A fourth set of wells without any peptide was used as a control. Two hours after infection, the concentration of gentamicin was lowered to 50 mg ml^{-1} while the concentration of MccJ25, $(KFF)_3K$, or McJ25-$(KFF)_3K$ was maintained for the rest of the experiment. Bacterial replication within eukaryotic cells was monitored at 2 and 6 h after infection. In order to do this, cells were lysed by using 1% Triton X-100, and the number of viable bacteria was determined by subsequent plating on LB agar. The results are represented as percentages of intracellular survival of bacteria obtained in each treatment compared with the control.

RESULTS

The peptide $(KFF)_3K$ sensitizes resistant strains to MccJ25. Table 2 shows that the antibiotic MccJ25 in the presence of 30 μg ml^{-1} $(KFF)_3K$ peptide inhibits all the strains analyzed except *Serratia marcescens*. For *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, the effect of the dual treatment was observed using 40 μg ml^{-1} $(KFF)_3K$ peptide. There was no inhibitory effect of MccJ25 on the resistant strains at lower concentrations of the $(KFF)_3K$ peptide (20 μg ml^{-1}) (data not shown). The sensitivity to MccJ25 of the susceptible *E. coli* strains, DH5α, MC4100, and AB259, was elevated in the presence of the $(KFF)_3K$ peptide, suggesting a non-MccJ25-specific uptake besides the specific one carried out by FhuA and SbmA. In addition, the resistant *E. coli* strains, SG303 *fhuA* (*fhuA* mutant) and EZE100 (*sbmA* mutant), became sensitive when exposed to the MccJ25-$(KFF)_3K$ treatment. The MIC of EZE100 to MccJ25 in the presence of $(KFF)_3K$ was approximately 8-fold lower than that obtained for the outer membrane mutant SG303 *fhuA*. In coincidence, the MIC of SG303 *fhuA* was the same as that of the *S.* Typhimurium strain with MccJ25-$(KFF)_3K$ treatment. This is an expected result given that this *E. coli* mutant is equivalent to this *Salmonella* strain in its difficulty in transporting microcin through the outer membrane (16). The dual treatment with MccJ25 and the $(KFF)_3K$ peptide reverts the microcin resistance of strain SBG231, which harbors a MccJ25-resistant RNAP (Table 2). This last result indicated that the MccJ25 affected the intracellular membrane target in strain SBG231. Previously, we showed that

TABLE 2. Effect of 30 μg ml^{-1} $(KFF)_3K$ on bacterial sensitivity to MccJ25

Strain or species	MIC (μM) of:	
	MccJ25	MccJ25-$(KFF)_3K$
E. coli		
DH5α	7.35	1.84
MC4100	7.35	0.46
AB259	3.67	0.23
SBG231	—[d]	58.7
SBG303	—	117.5
EZE100	—	14.7
S. Typhimurium		
14028s	—	117.5
SL3770	—	117.5
Serratia marcescens[a]	—	—
Citrobacter freundii[a]	—	7.35
Enterobacter cloacae[a]	—	7.35
Klebsiella pneumoniae[b]	—	58.7
Pseudomonas aeruginosa[c]	—	235[e]
Acinetobacter baumannii[a]	—	235[e]
Moraxella catarrhalis[a]	—	14.7

[a] Two different clinical isolates were evaluated with similar results.
[b] Three different clinical isolates were evaluated with similar results.
[c] Five different clinical isolates were evaluated with similar results.
[d] —, resistant at the last MccJ25 concentration tested (470 μM).
[e] 40 μg ml^{-1} $(KFF)_3K$ was used.

this target is evident in *E. coli* with high intracellular MccJ25 levels (2).

$(KFF)_3K$ treatment of all strains tested did not affect the bacterial growth at the concentrations used (30 and 40 μg ml^{-1}) (data not shown).

The susceptibilities of the strains to MccJ25/$(KFF)_3K$ treatment (Table 2) was quite varied. To elucidate whether this was due to inherent partial resistance of the membranes of some strains to $(KFF)_3K$, we evaluated if this permeabilizing peptide was able to equally enhance the susceptibilities of the strains to a reference antibiotic, such as rifampin. Table 3 shows that the $(KFF)_3K$ peptide enhanced the sensitivity to rifampin of all strains tested to various degrees (see "Fold increase in sensitivity"), suggesting a differential permeabilizing effect on the strains' membranes.

Taken together, these results suggest that the synthetic peptide makes MccJ25 uptake independent of both the FhuA and SbmA proteins. Moreover, the permeabilizing effect of the

TABLE 3. Effect of 30 μg ml^{-1} $(KFF)_3K$ on bacterial sensitivity to rifampin

Strain	MIC (μg ml^{-1}) of:		Fold increase in sensitivity[a]
	Rifampin	Rifampin-$(KFF)_3K$	
AB259	6.25	0.012	521
MC4100	6.25	0.024	260
EZE100	12.5	0.048	260
Citrobacter freundii	25	0.0061	4,098
Enterobacter cloacae	25	0.012	2,083
S. Typhimurium 14028s	6.25	0.0061	1,024
Pseudomonas aeruginosa	12.5	1.56	8
Acinetobacter baumannii	1.56	0.0061	256

[a] Ratio of rifampin MIC to that of rifampin-$(KFF)_3K$.

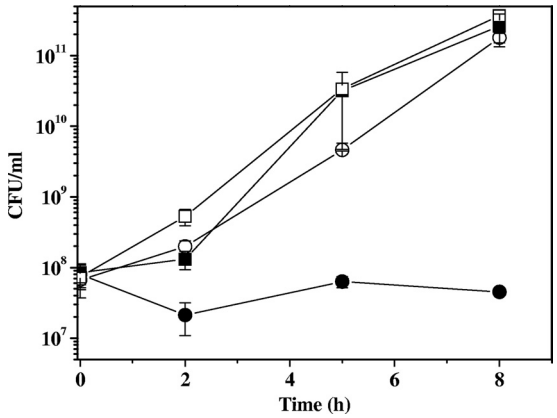

FIG. 1. Effects of treatment with MccJ25-$(KFF)_3K$ mix on bacteria viability. Bacterial viability was determined by CFU assay at different time points of bacterial growth in LB medium as a control (white squares) or in LB containing either 117.5 μM MccJ25 (white circles), 30 μg ml^{-1} $(KFF)_3K$ (black squares), or MccJ25-$(KFF)_3K$ (117.5 μM/30 μg ml^{-1}) mix (black circles). Error bars represent standard deviations from five independent experiments.

$(KFF)_3K$ peptide on the membranes of different strains appears to be disparate. For this reason, the differences in susceptibilities of MccJ25-resistant strains to combined treatment could be attributed to differential permeabilizing action of $(KFF)_3K$. However, it is not possible to rule out a differential MccJ25 action or internalization in each strain.

Effect of dual treatment, MccJ25 and $(KFF)_3K$, on the MccJ25 targets. We were interested in further studying the action of the MccJ25-$(KFF)_3K$ mix with *S.* Typhimurium, a facultative intracellular pathogen which is a model for evaluating the effect of antibiotics once the bacterium is internalized. For this reason, the *in vitro* action of the dual peptide treatment on this strain was first studied. As can be seen in Table 2, the *S.* Typhimurium 14028s strain was inhibited by MccJ25 (MIC, 117.5 μM) in the presence of $(KFF)_3K$. In order to quantify the effect of MccJ25-$(KFF)_3K$ treatment on MccJ25-resistant *S.* Typhimurium strain 14028s viability, colony counts were carried out at different times as described in Materials and Methods. Figure 1 shows that after 2 h of incubating cells with both peptides, MccJ25 (117.5 μM) and $(KFF)_3K$ (30 μg ml^{-1}), colony counts dropped from 10^8 to about 10^7 CFU/ml and remained unchanged until the end of the experiment. Meanwhile, in the absence of both peptides (control), the number of CFU/ml continued growing, reaching a normal colony count for this culture condition. Moreover, no effect was observed on bacterial viability when MccJ25 or the $(KFF)_3K$ peptide was individually added (Fig. 1).

We also wanted to know whether MccJ25 targets the *S.* Typhimurium RNAP and respiratory chain when $(KFF)_3K$ mediates its uptake. To this end, the effect of MccJ25 on *in vivo* RNA synthesis and oxygen consumption was explored in the presence of the $(KFF)_3K$ peptide. Our results demonstrated that RNAP and cell respiration were significantly inhibited in the simultaneous presence of both peptides while very little or no effect was observed when each peptide was individually added (Fig. 2 and 3). These data confirmed that the $(KFF)_3K$ peptide makes possible the entry of microcin, in an FhuA and

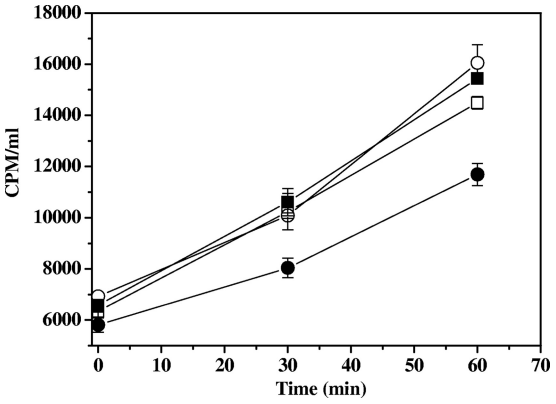

FIG. 2. Effects of MccJ25-$(KFF)_3K$ mix treatment on RNA synthesis. RNA synthesis was determined by monitoring [^{3}H]uridine incorporation at different time points of bacterial growth in the absence of peptides (white squares) or in the presence of 117.5 μM MccJ25 (white circles), 30 μg ml^{-1} $(KFF)_3K$ (black squares), or an MccJ25-$(KFF)_3K$ (117.5 μM/30 μg ml^{-1}) mix (black circles). Synthesis levels in each culture are represented as TCA-precipitable radioactive material (CPM/ml) retained on filters. Error bars represent standard deviations from five independent experiments.

SbmA-independent manner, reaching the intracellular concentration levels required to inhibit RNAP and respiratory chain targets and in consequence inhibiting bacterial growth (Fig. 1).

Effect of MccJ25/$(KFF)_3K$ dual treatment on *S.* Typhimurium replication inside eukaryotic cells. Once the *in vitro* effect of dual therapy on *S.* Typhimurium was characterized, the capability of MccJ25 to affect intracellular pathogen replication in the presence of $(KFF)_3K$ was investigated. To this end, we used RAW 264.7 macrophages infected with wild-type *S.* Typhimurium strain 14028s and subsequently treated with the MccJ25-$(KFF)_3K$ mix as described in Materials and Methods. After 2 h, the MccJ25-$(KFF)_3K$ mix was able to inhibit about 25% of the *Salmonella* replication compared with results for the control without peptides (Fig. 4). This effect was strongly increased with time, reaching 60% of the replication inhibition at 6 h (Fig. 4). These results demonstrated that MccJ25 can penetrate eukaryotic cells and inhibits pathogen replication when the $(KFF)_3K$ peptide is present. It is important to note that when the infected macrophages were treated with the $(KFF)_3K$ peptide alone, no effect on pathogen replication ability was observed (Fig. 4). Surprisingly, it was observed that MccJ25 alone was able to inhibit *Salmonella* replication 30% at 6 h (Fig. 4). This effect is currently under study.

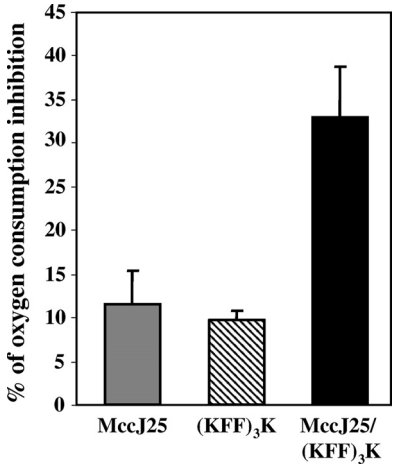

FIG. 3. Effects of treatment with MccJ25-$(KFF)_3K$ mix on oxygen consumption. Oxygen consumption of bacteria growing in the presence of MccJ25 (gray bar), $(KFF)_3K$ (striped bar), or MccJ25/$(KFF)_3K$ (black bar) was expressed as a percentage of the oxygen concentration of the control culture (without peptides). Data correspond to mean values of three independent experiments performed in duplicate. Error bars correspond to the standard deviations.

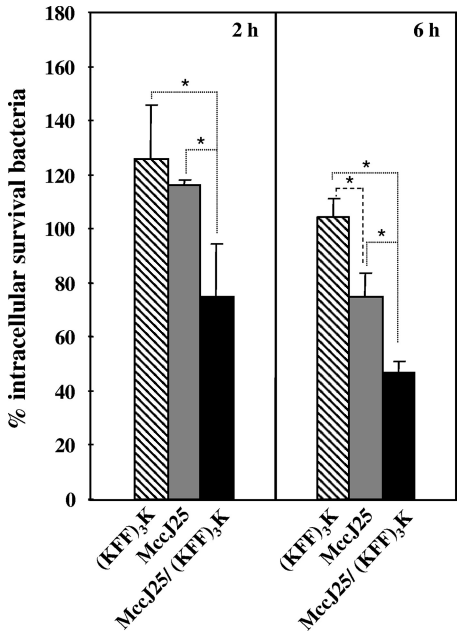

FIG. 4. Effect of MccJ25-$(KFF)_3K$ dual treatment on *Salmonella* Typhimurium survival within macrophages. RAW 264.7 macrophages were seeded in 24-well plates and grown for 24 h before bacterial infection with an overnight culture of *S.* Typhimurium strain 14028s. The bacteria infection was carried out at a multiplicity of infection of 10:1. The number of viable intracellular bacteria was determined at different times posttreatment with 117.5 μM MccJ25 (gray bar), 30 μg ml^{-1} $(KFF)_3K$ (striped bar), or MccJ25-$(KFF)_3K$ (117.5 μM/30 μg ml^{-1}) mix (black bar). Values are presented as the percentage of intracellular surviving bacteria in each treatment with respect to the control (without peptides). Error bars represent standard deviations from five independent experiments. *, statistically significant ($P < 0.01$).

DISCUSSION

Our long-term aim is the development of a useful antibiotic therapy with MccJ25. To this end, some issues, such as its *in vivo* efficacy, must be clarified by using the well-established murine model of infection with *S.* Typhimurium. However, this is hampered by the fact that *S.* Typhimurium is completely resistant to MccJ25. A relevant aspect of this work is the finding that strains that are naturally resistant to MccJ25, including *S.* Typhimurium strains, become sensitive when they are exposed to a simultaneous treatment with the antibiotic and the permeabilizing peptide $(KFF)_3K$.

It was previously demonstrated that the $(KFF)_3K$ peptide

sensitizes Gram-negative bacteria to hydrophobic and amphipathic antibiotics at levels similar to those seen with deacylpolymyxin B and polymyxin B (15). This effect would be produced by the interaction of $(KFF)_3K$ with the anionic LPS and the hydrophobic lipid A (15).

MccJ25 is a small hydrophobic peptide produced by an *E. coli* strain. Its uptake into sensitive bacteria requires the FhuA and SbmA proteins, located in the outer and inner membrane, respectively. This antibiotic is active against species related to the producer strain, like *Salmonella*, *Shigella*, and *E. coli*, while other enterobacterial strains were resistant to the microcin effect (16). Then, it is possible to say that MccJ25 shows a narrow spectrum of action. However, we previously showed that when the *fhuA* gene from *E. coli* was introduced into some resistant strains, these became hypersensitive to MccJ25 (16). Based on these results, it was postulated that the outer membrane is the principal barrier that MccJ25 should overcome to reach its targets. According to the above-mentioned data, the aim of this work was to expand the spectrum of action of MccJ25 by means of the outer membrane-permeabilizing peptide ($(KFF)_3K$).

We showed that the MccJ25 resistance of many clinical isolates could be overcome when they were exposed to dual treatment with MccJ25-$(KFF)_3K$ (Table 2). In this work, we mainly used a $(KFF)_3K$ concentration of 30 $\mu g\ ml^{-1}$, which was reported to be the minimal concentration with antibacterial activity (15). However, our assays did not show any direct antibacterial effect at concentrations of 30 and 40 $\mu g\ ml^{-1}$ $(KFF)_3K$. In fact, the MIC of the *E. coli* strain MC4100 and *S.* Typhimurium for this peptide was 50 $\mu g\ ml^{-1}$ (data not shown).

Different degrees of sensitization were obtained with the resistant strains evaluated. This could be attributed to a differential permeabilizing effect of $(KFF)_3K$ on the membrane of each strain. This possibility is considered likely because the $(KFF)_3K$ peptide dissimilarly increased rifampin susceptibilities of strains tested (Table 3). This allows us to propose a differential intrinsic resistance of some strains to the permeabilizing peptide. However, MccJ25 is an antibiotic that is recognized by both inner and outer membrane transporters and has specific targets with which it interacts. So, it is not possible to limit the explanation of the susceptibility variation with the combined treatment to the permeabilizing peptide effect. In this sense, an *sbmA E. coli* mutant was 8-fold more sensitive than an *fhuA E. coli* mutant when it was treated with the MccJ25-$(KFF)_3K$ mix, suggesting that the inner membrane barrier is permeabilized more efficiently by the $(KFF)_3K$ peptide. According to this, if the effect of $(KFF)_3K$ peptide is different in the two membranes, the degree of its action would depend on the ability of the outer or inner membrane transporters to uptake microcin in each strain. This is another potential explanation for the variability in the susceptibility with the combined treatment. The MICs of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* to combined treatment were comparable to that of an *E. coli* FhuA mutant. It is tempting to speculate that in these strains, the barrier would be the outer membrane, where the effect of the $(KFF)_3K$ peptide is less efficient. However, we cannot rule out that once inside, MccJ25 acts ineffectively on its targets in these strains. Under the MccJ25-$(KFF)_3K$ dual treatment, the MICs of *Citrobacter freundii*, *Enterobacter cloacae*, and *Moraxella catarrhalis*, strains less related to the microcin-producing one, were similar to that of the microcin-sensitive DH5α strain in the absence of the $(KFF)_3K$ peptide and to that of the *sbmA* mutant EZE100 in the presence of the $(KFF)_3K$ peptide (Table 2). This observation suggests that the only barrier with these strains is microcin uptake at the inner membrane level, which is facilitated by $(KFF)_3K$, allowing effective MccJ25 action on its intracellular targets. On the contrary, *Serratia marcescens* remained totally resistant with a dual treatment, suggesting the impossibility of MccJ25 targeting the RNAP and membrane respiratory chain of this strain.

It was also demonstrated that resistance of *S.* Typhimurium to MccJ25 could be overcome. Inhibition of both RNAP and oxygen consumption in this strain was observed when it was exposed to the dual therapy (Fig. 2 and 3). The effect on the targets in *Salmonella* was assessed using a microcin concentration corresponding to the MIC value (117.5 μM) together with the $(KFF)_3K$ peptide. The percentages of inhibition obtained for transcription and oxygen consumption were similar to those obtained for the DH5α strain with MccJ25 at a concentration equal to its MIC value (data not shown). Moreover, it was previously demonstrated that MccJ25 inhibited RNA polymerase and cell respiration in *S.* Typhimurium when this strain was transformed with a plasmid harboring the *E. coli fhuA* gene (16). The transformants obtained in this work were approximately 130-fold more sensitive to MccJ25 than the *E. coli* MC4100 strain. Consequently, a defect in MccJ25 transport through the SbmA protein of *S.* Typhimurium can be ruled out. We believe that the permeabilizing effect of $(KFF)_3K$ on the *S.* Typhimurium outer membrane could be less efficient than the overexpression of the *fhuA* gene. In fact, the sensitivity level of *S.* Typhimurium was considerably lower than that of this strain transformed with an *fhuA* plasmid (16) and was similar to that of an *E. coli* strain lacking FhuA (SG303 *fhuA*). However, the contribution of $(KFF)_3K$ to the MccJ25 effect was enough to produce a strong inhibition of *S.* Typhimurium viability (Fig. 1). On the contrary, no inhibition of RNA synthesis was observed when the peptides were assayed separately. Both MccJ25 and the peptide $(KFF)_3K$ inhibited cell oxygen consumption by 10%, but this effect was not enough to cause cell growth inhibition (Fig. 1). Apparently, a greater inhibitory effect on cellular respiration and/or RNAP inhibition would be necessary to get the antibiotic effect of microcin on growth. These results confirmed that the outer membrane is the main resistance barrier against microcin action. Moreover, it was demonstrated that this resistance barrier could be overcome in the presence of $(KFF)_3K$, allowing MccJ25 to enter the bacterial cell.

The capability of MccJ25 to penetrate macrophages and to inhibit pathogen replication in the presence of $(KFF)_3K$ was investigated. Unexpectedly, after 6 h of treatment, MccJ25 itself showed an inhibitory effect on intracellular pathogen replication (Fig. 4). This result could be attributed to modifications on the bacterial membrane due to the environment within macrophages. This effect is currently under study in our laboratory. Nevertheless, it is important to emphasize here that the presence of $(KFF)_3K$ not only enhanced the antibacterial activity of MccJ25 within macrophages, reducing the replica-

tion of *Salmonella*, but also decreased the time to achieve these inhibitory effects to 2 h after infection.

In summary, in this article, it is shown that the action spectrum of MccJ25 could be expanded by its combined administration with the permeabilizing peptide $(KFF)_3K$. These results constitute a potential contribution toward the use of this peptide antibiotic in therapeutic treatments.

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