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The antibacterial action of microcin J25: evidence for disruption of cytoplasmic membrane energization in *Salmonella newport*

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Abstract

Microcin J25 (MccJ25) is a cyclic peptide of 21 unmodified amino acid residues produced by a fecal strain of *Escherichia coli*. It has previously been shown that the antibiotic activity of this peptide is mainly directed to Enterobacteriaceae, including several pathogenic *E. coli*, *Salmonella* and *Shigella* strains. In this paper we show that MccJ25 acts on the cytoplasmic membrane of *Salmonella newport* cells producing alteration of membrane permeability, and the subsequent gradient dissipation, that initiate the inhibition of process, such as oxygen consumption. These results, taken together with our *in vitro* observations [Rintoul et al. (2000) Biochim. Biophys. Acta 1509, 65–72], strongly suggest that the disruption of the cytoplasmic membrane gradient is closely related to the bactericidal activity of MccJ25 in *S. newport*.

Keywords: Antimicrobial peptide; Microcin; Biological membrane

1. Introduction

Microcins form a miscellaneous group of low-molecular-mass peptide antibiotics produced by members of the family Enterobacteriaceae, mostly *Escherichia coli* strains. They are mainly active against bacterial genera or species phylogenetically related to the producing strains ([1,2]). Our laboratory is studying a new microcin, named J25 (MccJ25) ([3]). This plasmid-encoded antibiotic, synthesized and secreted into the culture medium by cells in the stationary phase of growth, is a cyclic peptide of 21 unmodified amino acid residues, *cycle*-(VGIGTPISFYGG-GAGHVPEYF) ([4]). Genetic studies and the nucleotide sequence of the plasmid region bearing the MccJ25 system revealed three genes (*mcjA,-B,* and *-C*) essential for MccJ25 production, and one gene (*mcj*D) that confer immunity to the antibiotic ([5,6]). In addition, the chromosomal *tolC* gene product is involved in the export of MccJ25 out of the producer cells ([7]). Previous work from our laboratory has shown that MccJ25 uptake by *E. coli* depends on the presence of FhuA, TonB, and SbmA cell envelope proteins ([8,9]).

As described previously, MccJ25 induces filamentation of *E. coli* in a non-SOS-dependent way ([3]). Microscopic examination of *E. coli* cultures after MccJ25 treatment showed that the cells gradually elongated, until long aseptate filaments were produced. Recently, we provided convincing evidence that RNA polymerase is the target for MccJ25 action in *E. coli*, provoking an impaired transcription of genes encoding cell division proteins ([10]).

MccJ25 is active against some pathogenic *Salmonella* and *Shigella* species ([3]). Portrait et al. ([11]) showed that MccJ25 produced by *E. coli* strains isolated from poultry intestines affect the growth of *Salmonella enteritidis*. Our previous studies ([3,4]) support the notion that MccJ25 has different modes of action on *E. coli* and *Salmonella newport*. Namely: (a) *S. newport* was much more

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Abbreviations: MccJ25, microcin J25; c.f.u., colony forming units; $DiSC_3[5]$, 3,3′-dipropylthiadicarbocyanine iodide

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sensitive to MccJ25 than *E. coli* strains (b) the minimal inhibitory concentration values (MIC) of the antibiotic for *S. newport* were independent of the culture media composition whereas for *E. coli* strains depend on the composition of growth media and (c) thermolysin-linearized microcin is still able to inhibit the *S. newport* but not *E. coli* strain. Studies reported here clearly indicate that MccJ25 disrupts the inner membrane gradient of the *S. newport* strain, inhibiting several processes essential for cell viability; on the other hand the peptide does not exert deleterious effect on the membrane of *E. coli*.

2. Materials and methods

2.1. Bacterial strains and media

The bacterial strains used were *E. coli* K-12 MC4100 [F$^-$*ara D139 Δ(argF-lac)205 λ$^-$ flB5301 relA1 rpsL150 deoc*1], harboring pTUC202, a low-copy-number recombinant plasmid with the MccJ25 system cloned ([6]), *E. coli* AB1133 [F$^-$*thr-1 ara14 leuB6 Δ(gpt-proA)62 lacY1 supE44 galK2 λ$^-$ rac nis64 rfbD1 rpsL31 KdgK51 xyl-5 mtl-l-1 argE3 thi-1* (*Sm^r*)] and a clinical isolate of *S. newport* The media used to grown the cells were LB rich and M63 minimal media ([12]), minimal medium supplemented with D-glucose (0.2%) and thiamine (1 μg ml^{-1}), and solid media contained 1.2% agar.

2.2. Microcin purification and susceptibility assays

MccJ25 was purified according to the procedure previously reported. This procedure yielded a preparation, which appeared homogeneous in two different systems of analytical RP-HPLC ([4]). All the susceptibility assays were done at 37°C with rotary shaking in LB medium. An Erlenmeyer flask (1000 ml), containing 400 ml of medium, was inoculated with 40 ml of an overnight culture of *E. coli* AB1133 or *S. newport*. Growth was followed by measuring the optical density at 600 nm (OD_{600}) of aliquots (2.0 ml) removed at the specified time intervals. MccJ25 was added at the indicated time. For bacterial viability assays, 100-μl aliquots were serially diluted in the same medium and plated in duplicate onto LB. After overnight incubation at 37°C the number of colony forming units (c.f.u.) was determined.

2.3. Determination of MICs

MICs were determined by the 2-fold serial dilution assay ([13]). Exponentially growing cultures of the test strains (OD_{600} = 0.4–0.5) were suitably diluted in the medium chosen for test, and 250-μl volumes (about 10^3 cells) were transferred to tubes containing 2-fold serial dilutions (250 μl) of antibiotic. Turbidity was read after 24 h of incubation at 37°C.

2.4. Dehydrogenase assays

The dehydrogenase activities of *E. coli* and *S. newport* membranes were determined at 37°C in 0.5 ml 50 mM phosphate (pH 7.5), following the reduction of the artificial acceptor MTT (50 μM ml^{-1}) to its formazan at 570 nm, in the presence of 0.5 mM NADH, 10 mM succinate or 10 mM D-lactate. The MccJ25 effect was studied by preincubation of the membrane (10 min at 37°C) with the antibacterial peptide (15 mM). Cytoplasmic membranes were obtained as described previously by Greenawalt ([14]).

2.5. O_2 consumption

Exponential phase cells (OD_{600} = 0.4–0.5) were harvested by centrifugation, washed and resuspended in buffer (50 mM sodium phosphate, pH 7.5). In three independent experiments 0.05 mM MccJ25 was added and the mixture was incubated at 37°C. At various times aliquots were removed, 10 mM D-glucose was added and the respiratory rate was measured. The average rate of respiration over the subsequent 5 min was measured polarographically with a Gilson oxygraph equipped with a Clark-type electrode in a closed stirred glass vessel of 2.0 ml volume at 37°C. The rate was normalized to that in the absence of MccJ25.

2.6. Cell membrane potential measurements

The membrane potential gradient was evaluated in bacterial cells with the potential-sensitive dye 3,3′-dipropyl thiacarbocyanine iodide ($DiSC_3$[5]) according to Béven [15]. Briefly, exponential growing cells were harvested, washed once, and resuspended in 5 mM HEPES buffer, pH 7.0, 50 mM D-Glucose, 5 mM EDTA, to obtain an OD_{600} = 0.1. Aliquots (2.0 ml) were incubated with 0.05 μM MccJ25 for 10 min at 37°C in a spectrofluorometer cuvette. Then, 0.4 μM $DiSC_3$[5] was added to the cuvette. The fluorescence signal at 675 nm was measured during 15 min using a SLM 4800 spectrofluorometer, exciting the sample at 622 nm. The decrease of the fluorescence intensity is directly proportional to the membrane potential ([16]).

2.7. Cell membrane permeabilization measurements

Cytoplasmic membrane permeabilization was determined by using the same dye-probe $DiSC_3$[5] [17]. The dye distributes between cells and the medium depending on the membrane potential gradient. Once inside the cell, it becomes concentrated and self-quenches its own fluorescence ([17]). *S. newport* was grown in LB at 37°C to exponential phase. The cells were collected by centrifugation and resuspended in 5 mM HEPES buffer, pH 7.0, 50 mM D-glucose, 5 mM EDTA, to obtain an OD_{600} = 0.1. The

cell suspension was incubated with 0.4 μM $DiSC_3[5]$ until the dye uptake was maximal (as indicated by a stable reduction of fluorescence). Then 100 mM KCl was added to equilibrate the cytoplasmic and external K^+ concentration [16]. The cell suspension previously loaded with $DiSC_3[5]$ (2.0 ml), was placed in a spectrofluorometer cuvette and the desired concentration of MccJ25 was added. The dye fluorescence increases were recorded as a function of time as described above. $DiSC_3[5]$-loaded cells in the absence of MccJ25 were used to subtract the background.

3. Results

3.1. Differential effect of MccJ25 on the growing culture of E. coli and S. newport

The effect of MccJ25 on exponential phase growing cells of *S. newport* and *E. coli* was studied (Fig. 1). The MccJ25 concentration added was 1.0 μM or 0.05 μM, corresponding to the MICs for *E. coli* AB1133 and *S. newport* strains, respectively. Addition of MccJ25 inhibited cell growth in both strains tested, but a decrease of cell viability was only observed in *S. newport* (the viable-cell count had dropped by three orders of magnitude after 4 h). Moreover, when the *S. newport* cells were removed from the culture medium (after 4 h of MccJ25 addition), washed and resuspended in fresh LB medium, cell viability was not recovered (data not shown). Previous results showed that for *E. coli* the block to division was relieved ([3]) under identical conditions.

3.2. Effects of MccJ25 on oxygen consumption and dehydrogenase activities

As can be observed in Fig. 2, 0.05 μM MccJ25 drastically inhibited the O_2 consumption of *S. newport*. After 30 min treatment, oxygen consumption levels were less than 20% compared to the cells that had not been treated. Microcin concentration higher than 0.5 μM did not induce any further depletion. On the other hand, oxygen consumption remained unaffected by exposure of *E. coli* to 1.0 μM MccJ25. The effect of MccJ25 on the activities of three dehydrogenases from *S. newport* and *E. coli* membrane is shown in Table 1. Experiments carried on with *S. newport* shown that 15 μM MccJ25 remarkably inhibited the activity of NADH and succinate dehydrogenases, both transmembranous associated enzymes ([18,19]). On the other hand, lactate dehydrogenase, a single-subunit enzyme that does not appear to have transmembrane elements ([19]), was slightly modified. Any of the three enzymes from *E. coli* membrane were not modified at the same conditions. These results clearly show a different susceptibility of dehydrogenase enzymes from *S. newport* and *E. coli* toward MccJ25 exposure, but can not establish a link with oxygen consumption neither membrane potential dissipation and the eventual bactericidal activity, since MccJ25 concentrations used differed considerably.

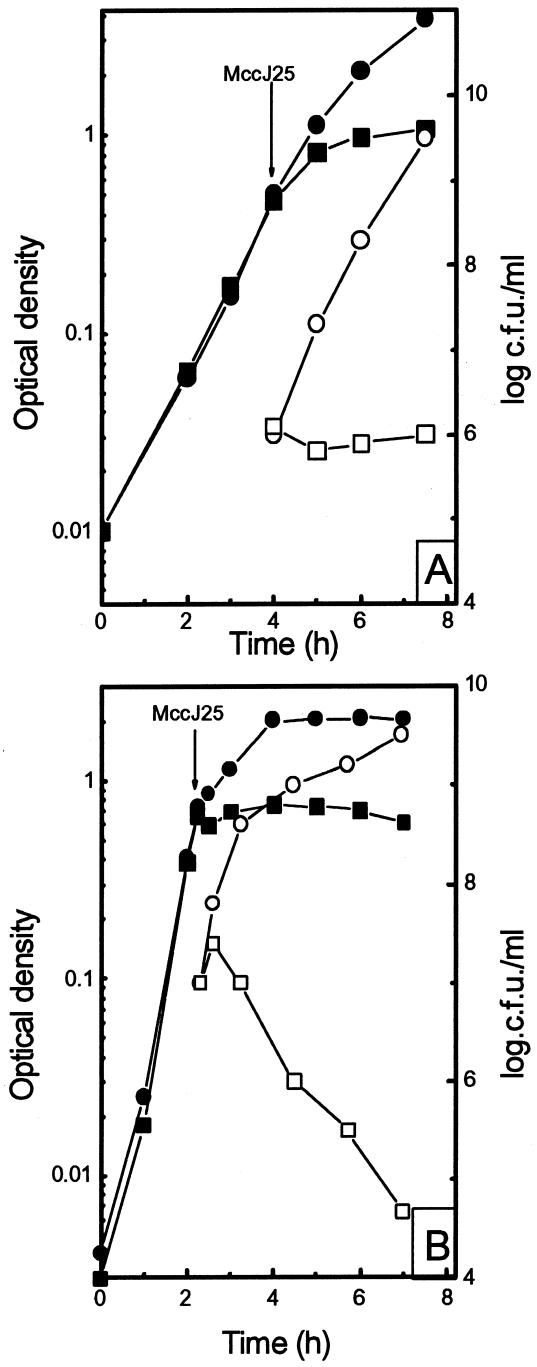

Fig. 1. Effect of MccJ25 on growth and viability of *E. coli* and *S. newport* Optical density (●,■) and cell viability (○) of *E. coli* AB1133 (A) and *S. newport* (B) in the presence (■,□) and in the absence (●,○) of MccJ25. Arrows indicate MccJ25 addition. The bacterial were growth as described in Section 2.

3.3. Effects of MccJ25 on the membrane potential

Since the respiratory function is dependent on an intact and functional cytoplasmic membrane, we decided to

Table 1
Effect of MccJ25 on the dehydrogenase activity from *S. newport* and *E. coli* membranes

Bacterial strain	Enzyme	Enzymatic activity (Δ570 nm min^{-1} (mg protein)$^{-1}$)		Inhibition (%)
		without MccJ25	with MccJ25	
S. newport	NADH dehydrogenase	1.75 ± 0.05	0.89 ± 0.05	49
	succinate dehydrogenase	2.01 ± 0.07	0.98 ± 0.08	52
	lactate dehydrogenase	0.83 ± 0.04	0.72 ± 0.06	13
E. coli AB1133	NADH dehydrogenase	1.56 ± 0.10	1.69 ± 0.10	0
	succinate dehydrogenase	2.11 ± 0.17	1.98 ± 0.28	0
	lactate dehydrogenase	0.93 ± 0.14	0.92 ± 0.06	0

The values are the means ± S.D. of the five experiments carried out separately by duplicate.

study the inner membrane integrity of *S. newport* in the presence of MccJ25. The alteration of the membrane potential was measured with the sensitive fluorescent probe $DiSC_3[5]$ in exponentially growing cells, as described in Section 2. The results are shown in Fig. 3. As can be seen, in the control experiment the fluorescence of $DiSC_3[5]$ shows an important decrease as a function of time, indicating that the *S. newport* membrane was polarized by incubation in a medium with glucose. However, when the cells were preincubated with MccJ25, the fluorescence decreased more slowly than in the control. The insert in Fig. 3 depicts the changes in fluorescence intensity after 15 min. The decrease was 40% for the control cells and only 25% for MccJ25-treated cells. Identical results were obtained by increasing the concentration of MccJ25 to values of 10-fold and 100-fold the MIC (not shown). No direct effect of MccJ25 on the fluorescence of $DiSC_3[5]$ was detected. When similar experiments were carried out using *E. coli*

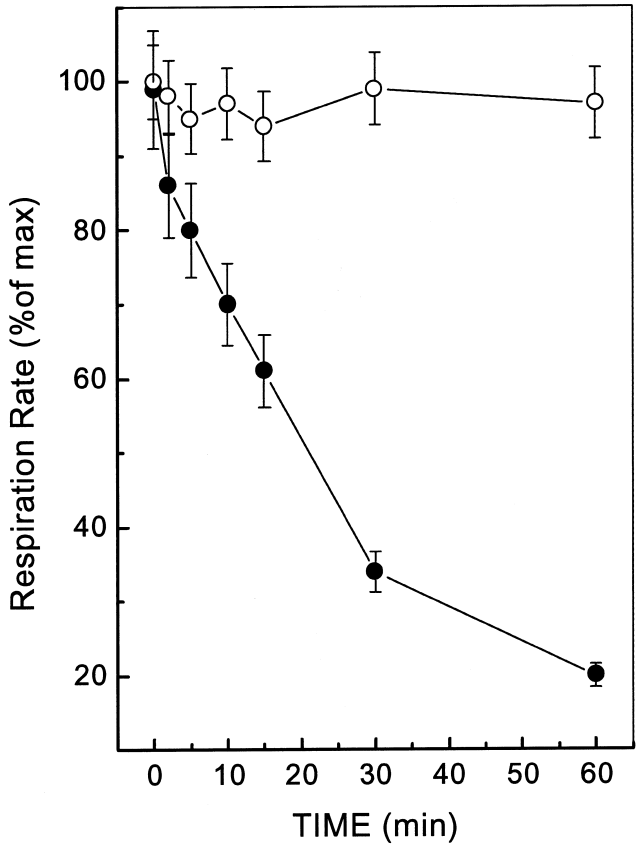

Fig. 2. Effect of MccJ25 on the oxygen consumption. *S. newport* (●) or *E. coli* (○) harvested in exponential phase and resuspended in 50 mM sodium phosphate, pH 7.5 were incubated at 37°C with 0.05 or 0.5 μM MccJ25 respectively. At various times aliquots were removed, 10 mM D-glucose was added and the respiratory rate determined as described in Section 2. The results were expressed as percent of the control measured in the absence of MccJ25. Error bars represent standard deviations from three experiments.

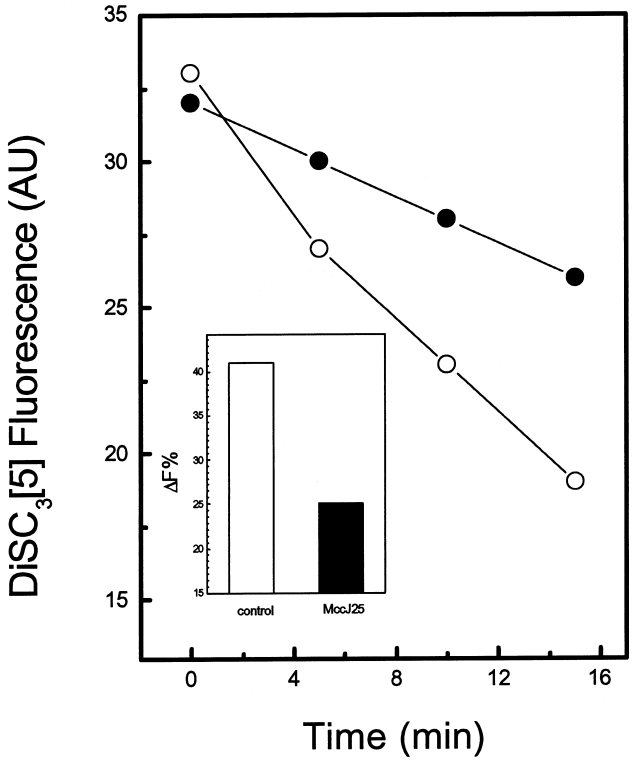

Fig. 3. Effect of MccJ25 on the cell membrane polarization. *S. newport* resuspended in 5 mM HEPES pH: 7.0, 50 mM D-glucose, 5 mM EDTA were incubated in the presence (●) and in the absence (○) of MccJ25 (0.05 μM). 0.4 μM $DiSC_3[5]$ was added and the fluorescence (excitation and emission wavelength, 622 and 675 nm respectively) was recorded as a function of time. Insert: percentage of fluorescence changes. The percentage membrane potential changes ($\Delta F\%$) was calculated according to $\Delta F\% = 100\ [(F_0 - F)/F_0]$. F_0, initial fluorescence; F, fluorescence value at 15 min.

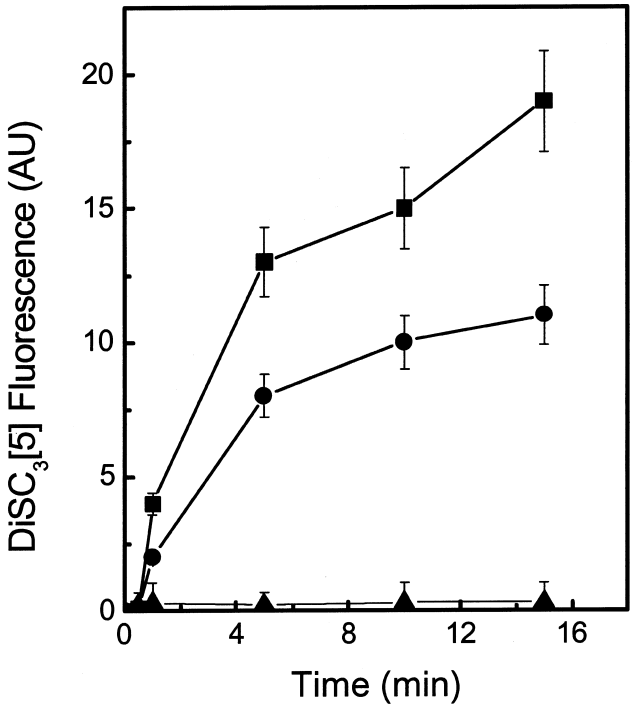

Fig. 4. MccJ25 effect on cell membrane permeability. *S. newport* suspended in 5 mM HEPES pH 7.0, 50 mM D-glucose, 5 mM EDTA were incubated with 0.4 μM $DiSC_3[5]$. Release of $DiSC_3(5)$-loaded cells in the absence (▲) and in the presence of 0.05 μM MccJ25 (■) or 0.037 μM valinomycin (●) was followed, as a function of time, measuring $DiSC_3[5]$ fluorescence (excitation and emission wavelength 622 and 675 nm respectively). Data points represent the means ± S.E.M. of three experiments done in triplicate.

strains instead of *S. newport* no alteration of the membrane potential was observed (results not shown).

3.4. Effects of MccJ25 on membrane permeability

To confirm the results shown in Fig. 3 and to rule out that they were due to a loss in cell viability during MccJ25 treatment, the following experiments were performed. Cells were pre-loaded with DiSC[5] previous to the addition of MccJ25. After the addition of MccJ25 to dye-loaded cells an increase in fluorescence appeared (Fig. 4). The immediate increase in fluorescence suggested that the effect was not due to cell death. As noted by Wu et al. ([17]) the increased fluorescence correlates with the membrane potential dissipation and membrane permeability enhanced. In parallel experiments, the K^+ ionophore valinomycin, known by its ability to change the biological membrane potential, was used as a positive control. In *E. coli* cells the action of MccJ25 on the membrane permeability evaluated by this methods did not showed any alteration (results not shown).

4. Discussion

The results presented in this study clearly show a differential effect of MccJ25 on *S. newport* and *E. coli*. We did not tested other *Salmonella* subspecies, and this aspect is under investigation now. In *S. newport* cells MccJ25 induces a rapid inhibition of oxygen consumption. Concomitantly, we observed the inhibition of succinate and NADH dehydrogenases, two integral membrane enzymes, while only a slight effect was obtained on D-lactate dehydrogenase, a peripheral membrane enzyme. These effects correlate well with a remarkable and irreversible decrease in cell viability. We investigated the possibility that this microcin affected the membrane permeability. In fact, our results indicate that MccJ25 depolarizes the cytoplasmic membrane. When we investigated the alteration of the membrane potential, membrane permeability and oxygen consumption by MccJ25 using *E. coli* strains instead of *S. newport* these parameters were unaffected.

Based on these and our previous findings obtained with artificial membranes ([20]), and taking into account the time-correlated effects, we hypothesize that MccJ25 exerts a strong effect on *S. newport* by first inducing a change in the membrane permeability. This, in turn, would lead to a collapse of the electrochemical gradient and the subsequent loss of the cell energetic charge. As a result cells would stop growing and finally die. It is uncertain, however, whether this membrane perturbation is the unique effect and responsible of the antimicrobial activity of this peptide on *S. newport*. It is possible that an additional effect on the cell metabolism is produced. At the present, we are not able to discriminate between these possibilities and further investigations are carried out in our laboratory.

The increased susceptibility of *S. newport* to microcin could be explained by an increased permeability of the outer membrane of *S. newport* compared to that of *E. coli*. However, this hypothesis it is difficult to explain since we have not detected changes of MccJ25 sensitivity of a wild-type [D21 (proA23 lac-28 tsx-81 trp-30his-51 rpsL173 ampCp-1)] and its isogenic series of well-characterized *E. coli rfa* mutants [D21e7 (D21 *rfa*-1) and D21f2 (D21 *rfa-1 rfa-3*1)], which do not synthesize complete wall lipopolysaccharide but synthesize core lipopolysaccharide, and express the deep rough and permeable phenotype [21].

Aside from its potential as a human therapeutic agent, MccJ25 may be effective as a food preservative, since the species *Salmonella* is a frequent cause of human food poisoning. Therefore, understanding the molecular basis of MccJ25 action on these pathogenic bacteria should help to clear the path for these commercial applications.

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