Cytochromes bd-I and bo3 are essential for the bactericidal effect of microcinJ25 on Escherichia coli cells

A.E. Galván, M.C. Chalón, Schurig-Briccio, R.A. Salomón, C.J. Minahk, R.B. Gennis, A. Bellomio

PII: S0005-2728(17)30158-5
DOI: doi:10.1016/j.bbabio.2017.10.006
Reference: BBABIO 47845

To appear in:

Received date: 11 April 2017
Revised date: 4 October 2017
Accepted date: 27 October 2017

Please cite this article as: A.E. Galván, M.C. Chalón, Schurig-Briccio, R.A. Salomón, C.J. Minahk, R.B. Gennis, A. Bellomio , Cytochromes bd-I and bo3 are essential for the bactericidal effect of microcinJ25 on Escherichia coli cells. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Bbabio(2017), doi:10.1016/j.bbabio.2017.10.006

Cytochromes *bd*-I and *bo*$_3$ are essential for the bactericidal effect of microcinJ25 on *Escherichia coli* cells

Galván, A.E.[1]; Chalón, M.C.[1]; Schurig-Briccio[2], L.A.; Salomón R.A.[1]; Minahk, C.J.[1]; Gennis, R.B[2] and Bellomio, A[1*].

1 Instituto Superior de Investigaciones Biológicas (INSIBIO, CONICET-UNT) and Instituto de Química Biológica "Dr. Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco 461, T4000ILI – San Miguel de Tucumán. Argentina

2 Department of Biochemistry, University of Illinois, Urbana, IL 61801, USA.

* Corresponding author:

Augusto Bellomio

Chacabuco 461

San Miguel de Tucumán (T4000ILI), Argentina

Fax: +54 0381 4248921

Tel: +54 0381 4248921

E-mail: augustobellomio@fbqf.unt.edu.ar

ABSTRACT

Microcin J25 has two targets in sensitive bacteria, the RNA polymerase, and the respiratory chain through inhibition of cellular respiration. In this work, the effect of microcin J25 in *E. coli* mutants that lack the terminal oxidases cytochrome *bd*-I and cytochrome bo_3 was analyzed. The mutant strains lacking cytochrome bo_3 or cytochrome *bd*-I were less sensitive to the peptide. In membranes obtained from the strain that only expresses cytochrome *bd*-I a great ROS overproduction was observed in the presence of microcin J25. Nevertheless, the oxygen consumption was less inhibited in this strain, probably because the oxygen is partially reduced to superoxide. There was no overproduction of ROS in membranes isolated from the mutant strain that only express cytochrome bo_3 and the inhibition of the cellular respiration was similar to the wild type. It is concluded that both cytochromes *bd*-I and bo_3 are affected by the peptide. The results establish for the first time a relationship between the terminal oxygen reductases and the mechanism of action of microcin J25.

Keywords: MICROCIN J25, RESPIRATORY CHAIN, CYTOCHROME, ROS.

Abbreviations: MccJ25: Microcin J25; MIC: Minimum inhibitory concentration; RNAP: RNA polymerase. GME: glycine methyl ester, EDAC: 1-ethyl-3-[3-(dimethyl amino) propyl]-carbodiimide hydrochloride. MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide).

1. Introduction

Microcin J25 (MccJ25) is a class I, low molecular weight microcin (Accession: BAC015) [1,2]. Class I microcins are post-translationally modified bacteriocins produced by Gram-negative bacteria [3]. MccJ25 has a peculiar structure consisting of a ring of eight amino acids (formed by a lactam bond between amino acids 1 and 8) and a C-terminal tail of 13 amino acids folded upon itself that passes through the ring [4–6]. This peptide is naturally produced by the strain AY25 of *Escherichia coli*. It is active against foodborne pathogenic bacteria, such as *Shigella* spp., *E. coli* O157:H7 and *Salmonella* spp. [1,7,8]. MccJ25 is recognized by a ferric hydroxamate uptake receptor, called FhuA [9,10], which obtains energy from the electrochemical gradient generated in the inner membrane. This energy is transduced to FhuA by the protein complex TonB-ExbB-ExbD [11]. Once in the periplasm, MccJ25 must pass through the inner membrane, and this process involves the SbmA protein [11,12]. Once in the cytoplasm, MccJ25 inhibits the RNA polymerase (RNAP) of sensitive bacteria [13]. The peptide occludes the RNAP secondary channel, preventing the entry of ribonucleotide precursors to the active site of the enzyme [14]. MccJ25 interacts moderately weakly with RNAP with an apparent Ki of 20 μM at a saturating NTP concentration [15].

MccJ25 has a second independent mode of action due to an enhanced production of reactive oxygen species (ROS) by a mechanism yet to be determined. The overproduction of superoxide is accompanied by inhibition of components of the aerobic respiratory chain [16]. The inhibitory effect of MccJ25 on *E. coli* respiration can be readily observed when its receptor, FhuA, is overexpressed, allowing more MccJ25 to enter the cell [16]. A chemically modified version of MccJ25, called MccJ25-GA, has been shown to selectively act on the respiratory chain but not on the RNAP. This derivative was obtained by amidating the C-terminal Gly^{21} carboxyl group with L-glycine methyl ester [17]. The modified microcin can be used to selectively examine the effects of the antibiotic in the absence of any perturbation to RNAP.

The effects of MccJ25 on intact rat heart mitochondria have also been studied [18]. As observed with bacteria, MccJ25 increases the production of superoxide which, in turn, results in triggering the opening of the mitochondrial transition pore [19], the release of cytochrome *c* [19] and inhibition of enzymes of the respiratory chain [20]. The mechanism remains to be worked out but is presumably related to what also occurs in *E. coli.*

In the current work, the activities of MccJ25 and MccJ25-GA are examined in intact cells and in membranes prepared from strains of *E. coli* that have mutations in one or more of the respiratory oxygen reductases present in the organism. *E. coli* has three respiratory oxygen reductases: cytochrome bo_3 and two closely related *bd* cytochromes, *bd*-I and *bd*-II [21]. These enzymes oxidize quinol in the cytoplasmic membrane and reduce O_2 to water and each directly contributes to generating the proton motive force, which is used in ATP synthesis. Cytochrome bo_3 predominates under high aeration growth conditions and is part of a large family of heme-copper respiratory oxidases with homologs in almost all bacteria as well as in mitochondria [22–24]. The *bd*-type cytochromes are also membrane-bound heme proteins, but lack copper and are not phylogenetically related to the heme-copper oxidoreductases. The *bd*-type oxygen reductases are found in a number of bacterial pathogens [25]. Cytochrome *bd*-I from *E. coli* is expressed under low oxygen tension and was the first *bd*-type oxygen reductase to be biochemically isolated and characterized [22]. One of the physiological functions of cytochrome *bd* is to act as an oxygen scavenger, thereby protecting cells from ROS damage, facilitating bacterial colonization of ecological niches at low O_2 tension [21]. Cytochrome *bd*-II is closely related to cytochrome *bd*-I and also has a very high affinity for O_2, but the two *bd*-type oxygen reductases are expressed under different growth conditions [26,27]. In this work, we demonstrate that the effects of MccJ25 on *E. coli* cells and membranes depend on what oxygen respiratory reductases are present.

2. Material and methods

2.1 Strains and plasmids

Bacterial strains and plasmids used in this work are shown in Table 1. The source or reference and the relevant features are highlighted.

Table 1 Strains and plasmids.

Strain	**Genotype**	**Reference**
AB259	HfrH*supQ80 λ. relA1 spoT1 thi-1*	CGSC
C43	F^-ompT gal dcmhsdS_B($r_B^-m_B^-$)(DE3)	[28]
C43 Δ*bd*-I	C43 (DE3) *cydABX*	Myat Lin and R.B. Gennis
C43 Δbo_3	C43(DE3) *cyoABCDE::kan*	[29]
CBO	C43 (DE3) *cydABX, appBCX*	[30]
CBDI	C43(DE3) *cyoABCDE::kan,appBCX*, Km^r	Myat Lin and R.B. Gennis
Salmonella enterica serovar Newport	Clinical isolate	FBQF-UNT
Plasmids	**Description**	**Reference**
pTUC200	pBR322 *mcjABCD*$^+$, Ap^r	[31]
pTUC203-6	pACYC184 184 *mcjBCD*$^+$*mcjA::Cm*, Cm^rKm^r	[32]
pGC01	pBR322 *fhu*A^+, Ap^r	[33]

CGSC, *Escherichia coli* Genetics Stock Center; Km^r, kanamycin resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; FBQF-UNT, Facultad de Bioquimica, Quimica y Farmacia, Universidad Nacional de Tucuman.

The presence of the *fhuA* and *sbmA* mutations was verified by the resistance to colicin M (*fhuA*), bacteriophage T5 (*fhuA*) and microcin B17 (*sbmA*) [34–36].

2.2 Culture media and reagents

Bacterial strains were cultured in LB or M9 (Sigma-Aldrich, USA) [37]. The M9 minimal medium was supplemented with 0.2 % D-glucose, 0.2 % casein enzymatic hydrolyzate (Sigma-Aldrich, USA), vitamin B1 (1 µg/ml) and $MgSO_4$ (1 mM) after autoclaving. Thiamine (vitamin B1) was sterilized by filtration. To prepare solid media, 1.5% (w/v) agar (Sigma-Aldrich) was added.

2.3 MccJ25 and MccJ25-GA purification

MccJ25 was purified according to the procedure described by Blond *et al* [38]. Cultures from MccJ25 producing strain were grown in Erlenmeyer flasks containing 1 liter of supplemented M9 medium. After 36 hours, the cultures were centrifuged at 6,000 *x g* for 10 minutes. The cell-free filtrates were passed through preparative C8 cartridges (Phenomenex), and MccJ25 was eluted with increasing concentrations of methanol. Then, a semi-preparative reverse phase HPLC (Waters µBondapack™ C18 – 10 µm) was performed using water-0.1% TFA and acetonitrile-0.1% TFA as solvents A and B, respectively. The activity of eluted fractions was determined using the MccJ25 sensitive strain *S.* Newport.

The amidation of the MccJ25 carboxyl group was carried out according to a modification of the previously described procedure [17,39]. To separate the modified MccJ25-GA from the native peptide, an anion exchange chromatography (FPLC - Pharmacia Biotech) was performed using a Protein-Pak™ DEAE 8HR (Waters AP-1) column. A linear gradient between 0 and 0.5 M NaCl was performed in 10 mM HEPES buffer pH 8. MccJ25-GA has no negative charge upon amidation of the C-terminal carboxyl group. Hence, it can be eluted in the void volume, while native MccJ25 interacts with the column and is retained. The peptides identities were confirmed by mass spectrometry (Additional Figure 1).

2.4 Determination of peptide concentration

In order to prepare stock solutions, the lyophilized peptides were dissolved in methanol. The concentration of the stock solution was spectrophotometrically determined at 278 nm (Beckman DU7500) using a molar extinction coefficient (ε) of 3340 cm^{-1} M^{-1} [40].

2.5 Minimal inhibitory concentration test

The sensitivity of the different strains to MccJ25 and MccJ25-GA was determined by a modified critical dilution method [41]. A 0.5 mM solution of either MccJ25 or MccJ25-GA was successively diluted with 0.1% Tween 80. Then, 10 µl of each dilution was spotted onto an M9 agar plate. The plate was then overlaid with 4 mL soft agar (0.6%), inoculated with approximately 10^7 cells of the test strain. After overnight incubation at 37°C, the plate was examined for growth inhibition. The MIC was taken as the concentration of peptide present in the last dilution capable of producing a halo of inhibition.

2.6 Viability assay

Strains were transformed with pGC01. Then, exponentially growing cultures were exposed to 20 µM MccJ25 or methanol (control) for 30 minutes and aliquots were successively diluted in M9.One hundred microliters of each dilution were spread on LB plates with ampicillin. The CFU/ml were counted after overnight incubation.

2.7 Isolation of bacterial membranes

Bacterial membranes were obtained as previously described by Evans [42]. Cells were grown in LB medium (OD_{600nm} 0.8), harvested by centrifugation (6,000 *x g*, 4 °C 10 min) and resuspended in 20 mM Tris-HCl, 1 mM $MgCl_2$ pH 7.5 (buffer T). Afterward, they were broken by passage through a French press (Thermo French Press). Cell debris was separated by centrifugation (6,000 *x g*, 4 °C, 10 min). The bacterial membranes were obtained by ultracentrifugation at 76,000 *x g* (Beckman Coulter - USA) for 60

minutes at 4°C. Finally, the protein concentration was determined by the Lowry method [43] and fractions were stored at -70 °C until use.

2.8 Oxygen consumption

The average rate of cell respiration was measured at 37°C with constant stirring with a Clark Type polarographic oxygen electrode (Oroboros oxygraph-2k). Bacterial cultures were grown in LB to an OD_{600nm} of 0.8, harvested by centrifugation at 6,000 x *g* for 10 min and washed twice with M9 medium. The cells were resuspended to an OD_{600nm} of 0.2 in LB. The cultures were preincubated with MccJ25 and MccJ25-GA (20 µM) during 30 minutes at 37 °C. Glycerol [0.2 % (w/v)] was used as carbon source.

2.9 Determination of NADH dehydrogenase and oxidase activities

NADH dehydrogenase and oxidase activities were determined as previously described [44]. The reaction mixture containing the membrane extract (10 µg/ml protein), MTT (50 mg/ml) and MccJ25 (20 µM) was preincubated for 10 minutes at 37 °C. A control without peptide was included. The substrate (0.5 mM NADH) was added and MTT reduction was followed at 570 nm in the presence of 6 mM KCN. For NADH oxidase activity, the NADH consumption was measured at 340 nm without MTT and KCN. The polarographic determination of *E. coli* C43 membranes NADH oxidase activity was carried out using a Clark type electrode to measure oxygen concentration. The reaction mixture in 50 mM sodium phosphate buffer pH 7.4 containing the membrane extract (10 µg/ml protein) and MccJ25 (or the same volume of methanol for control) at a final concentration of 20 µM was preincubated for 10 minutes in the cell of the oxygraph under magnetic stirring at 37 °C (Oroboros oxygraph-2k). The volume of methanol was never greater than 0.7% of the total volume of the reaction. The cell was capped and the reaction started with the addition of 10 mM NADH. When necessary, 150 IU Mn-SOD, 425 IU bovine liver catalase or 6 mM KCN were added.

2.10 Measurement of ROS

The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes-Invitrogen) was used for measuring ROS production. The diacetate groups were eliminated by treatment with 0.01 N NaOH for 30 minutes at room temperature. The resulting DCFH was neutralized with 25 mM NaH_2PO_4 (pH 7.4) and stored on ice. Purified cell membranes (0.1 mg/ml) were mixed with 3 mM DCFH and 20 µM MccJ25. The reaction was initiated by adding 10 mM sodium succinate or 100 µM reduced quinone as substrate. The assay was conducted in the absence and presence of superoxide dismutase (SOD). Fluorescence emission at 37°C was recorded as a function of time in a Perkin-Elmer LS-55 Fluorescence Spectrometer. Excitation and emission wavelengths were set at 485 nm and 520 nm, respectively [45,46].

The reduced quinone (QH_2) was prepared from the commercial ubiquinone Q_1 (Sigma-Aldrich, USA) by reduction with sodium borohydride [47]. Ubiquinone Q_1 was dissolved in 200 µl of anhydrous ethanol to achieve a 10 mM concentration. Then, 100 µl of 1.2 N HCl and a few grains (about 5 mg) of $NaBH_4$ were added until a completely colorless and reduced solution was obtained. The extraction of QH_2 was carried out adding 400 µl of cyclohexane. The organic phase was transferred quickly to another clean glass tube. When the cyclohexane was dried under N_2, the QH_2 was dissolved in 200 µl of anhydrous ethanol. The concentration of reduced quinone was measured spectrophotometrically using the $\varepsilon_{290\ nm} = 4\ mM^{-1}\ cm^{-1}$.

2.11 Effect of ascorbic acid on MccJ25 sensitivity

The ascorbic acid effect was studied by the antibiotic disk diffusion method [48]. M9 plates supplemented with 5 mM ascorbic acid were employed and a bacterial lawn was prepared as mentioned above. Whatman filter discs containing MccJ25 at different concentrations were deposited onto the lawn. The plates were incubated overnight at 37°C.

3. RESULTS

3.1 Effect of MccJ25 on *E. coli* cytochrome mutant strains

To help clarify the requirements for the observed effects of MccJ25 on *E. coli*, the antibiotic was examined with four different C43 mutant strains: 1) a mutant lacking cytochrome *bd*-I; 2) a mutant lacking cytochrome bo_3; 3) a mutant lacking both cytochrome bo_3 and cytochrome *bd*-II (CBDI); and 4) a mutant lacking both cytochrome *bd*-I and cytochrome *bd*-II (CBO).

Figure 1 shows that the MICs for the different strains do not differ significantly (0.24-0.48 µM, clear halos), except for strain CBO (Δ*bd*-I/Δ*bd*-II) which is completely resistant to both MccJ25 and MccJ25-GA. This resistance was traced to a mutation in *fhuA*, which encodes FhuA, the ferrichrome-iron receptor of *E. coli* that also acts as the outer membrane receptor/transporter used by MccJ25. FhuA is required for the antibiotic to gain entry to the cells to attain sufficient intracellular concentration to be effective [16]. This was confirmed by transforming all the strains with the plasmid pGC01, a pBR322 derivative which overexpresses FhuA [33]. The MIC of the Δ*bd*-I/Δ*bd*-II double mutant drops from >500 µM to 15 nM when *fhuA* is overexpressed. For the WT strain, overexpression of *fhuA* also results in a reduction of the MIC from 240 nM to 8 nM (Figure 1) and the viability count decreases 3.14 log units (Figure 2). A similar reduction of the MIC is not observed in either of the two mutant strains in which cytochrome *bd*-II has not been deleted (strain Δbo_3 and strain Δ*bd*-I).

3.2 MIC determination for MccJ25-GA

MccJ25-GA is an amidated variant of MccJ25 and has lost its ability to interact with the *E. coli* RNAP [17]. As shown in Figure 1, if FhuA is not overproduced, the MIC value is ≥125 µM for all of the strains including the WT. The primary target of MccJ25 is clearly RNAP. In the WT strain, overproduction of FhuA results in lowering the MIC value of MccJ25-GA from 125 µM to 3.9 µM. The MIC of the MccJ25-GA for strains that can only produce cytochrome bo_3 (Δ*bd*-I/Δ*bd*-II) or can only express cytochrome *bd*-I

(Δ*bo*$_3$/Δ*bd*-II) suggest that the MccJ25-GA affect the respiratory chain equally well when either cytochrome *bo*$_3$ or cytochrome *bd*-I is the sole oxygen reductase present. The most dramatic result observed is that the MIC is >500 µM even when *fhuA* is overexpressed in the strain from which the operon encoding cytochrome *bo*$_3$ has been deleted. Sensitivity to MccJ25-GA is restored when the operon encoding cytochrome *bd*-II is also deleted, i.e., Δ*bo*$_3$/Δ*bd*-II. In other words, if the presence of either cytochrome *bo*$_3$ or cytochrome *bd*-I confer sensitivity to MccJ25-GA, then the ability of the strain to also express cytochrome *bd*-II can confer some degree of resistance to MccJ25-GA, with the exception of the WT strain, which remains sensitive (MIC 3.9 µM).

3.3 Effect of MccJ25 on *E. coli* respiration

Inhibition of the respiratory chain is one of the causes for MccJ25 toxic effect on *E. coli* and *S.* Newport [49,16]. However, overexpression of the outer membrane transporter, FhuA, is required for observing the MccJ25 effect in *E. coli* [16]. In fact, no significant inhibition of oxygen consumption was observed when *fhuA* was only expressed from the single-copy chromosomal gene (data not shown). Figure 3 shows the inhibition of respiration of pGC01-transformed cells treated with 20 µM MccJ25 compared to the untreated control. In mutant strains having cytochrome *bo*$_3$ (Δ*bd*-I and CBO) the inhibition does not differ from that seen in the WT strain (> 20 % inhibition). By contrast, strains having cytochrome *bd*-I are less affected. The Additional Figure 2 indicates no inhibition for Δ*bo*$_3$ strain and figure 3 shows 12.6 ± 3.2 % for the CBDI strain (Δ*bo*$_3$/Δ*bd*-II). Similar results were obtained with MccJ25-GA: parental strain, 20.9 ± 2.9 %; Δ*bd*-I, 17.4 ± 4.4 %; and Δ*bo*$_3$, no inhibition (Additional Figure 3).

3.4 Effect of MccJ25 on *E. coli* NADH dehydrogenase and oxidase activities

NADH dehydrogenase activity in *E. coli* membranes was determined by three different approaches: 1) reduction of MTT in the presence of KCN. Electrons flow directly from the reduced quinones to the artificial acceptor, MTT; meanwhile, terminal oxidases are blocked with cyanide; 2) oxidation of NADH. Absorbance at 340 nm is recorded as a

measure of NADH concentration; 3) oxygen consumption. This activity includes the flow of electrons from NADH dehydrogenases to the terminal oxidases.

In all the isolated membranes tested, no inhibition of NADH dehydrogenase activity by MccJ25 is observed in the presence of KCN and MTT (Additional Figure 4). However, when absorbance at 340 nm was determined a small inhibition of NADH oxidation is noted with MccJ25 (Figure 4). The values are similar to those previously reported [16]. The highest inhibition is obtained with membranes isolated from the double mutant CBDI (23.1 ± 7.2 %).

Since MccJ25 induces ROS production, we evaluated the effect of the peptide on the oxygen consumption of membranes obtained from the WT strain. We used NADH as a substrate of the respiratory chain in the absence or presence of SOD and catalase. These enzymes are involved in the detoxification of superoxide and hydrogen peroxide, respectively, as shown in equations 1 and 2.

$$2\,H^+ + O^{\cdot -}_2 + O^{\cdot -}_2 \longrightarrow H_2O_2 + O_2 \qquad \text{Eq. 1}$$

$$2\,H_2O_2 \longrightarrow O_2 + 2\,H_2O \qquad \text{Eq. 2}$$

As can be seen in Figure 5A and 5B, O_2 consumption rate decreases to about half of the value without microcin. In the presence of *E. coli* Mn-SOD the rate of oxygen consumption diminishes (Figure 5C), the slope decreases even more with MccJ25, i.e. approximately one-quarter of that obtained without MccJ25 (Figure 5D). Moreover, when catalase is added to the reaction medium containing MccJ25 and SOD the rate of O_2 consumption is almost void (Figure 5D). The production of O_2 in the detoxification reactions of SOD and catalase could explain these results. Importantly, the presence of

cyanide in the medium completely abolishes O_2 consumption (see Additional Figure 5), indicating that ROS formation occurs through terminal oxidases.

3.5 Superoxide overproduction induced by MccJ25 in isolated membranes

Herein, the production of ROS by microcin was studied in membranes obtained from different terminal oxidase mutants. For this purpose, DCFH probe, which is capable of detecting hydrogen peroxide, superoxide and hydroxyl radical was employed [50–53]. *E. coli* Mn-SOD was used as an indicator of superoxide formation. The difference in DCFH oxidation (fluorescence intensity) in the absence *vs.* presence of SOD corresponds to the produced superoxide (Additional Figure 6). Figure 6 shows that superoxide generation increases in the presence of MccJ25. It is important to note that the highest increment (242 % for CBDI) is obtained using membranes isolated from the strain which only expresses cytochrome *bd*-I. In the absence of cytochrome *bd*-I (with membranes of *E. coli* Δ*bd*-I and *E. coli* CBO), the production of ROS is lower, 85 % of increase and no significant variation, respectively. To demonstrate that ROS formation occurs downstream of the dehydrogenases, the electrons were delivered to cytochromes from reduced quinone instead of succinate as a substrate for the respiratory chain. The overproduction of ROS was evidenced by an increase of ~500% fluorescence in the presence of MccJ25 with respect to the control in membranes obtained from wild type strain (Figure 7).

3.6 Ascorbic acid effect on MccJ25 antimicrobial activity

Increase in antibiotic resistance by the addition of an antioxidant indicates the involvement of ROS in its mechanism of action [54]. To verify if this is the case for MccJ25, the sensitivity to the peptide was evaluated with ascorbic acid added directly to M9-agar plate. In all the tested strains, inhibition halos around filter paper disks with MccJ25 turned out to be smaller and more turbid in the presence of the antioxidant

(Figure 8). This result confirms that ROS are important in the mechanism of action of the peptide.

4. DISCUSSION

The aim of this work is to study the importance of *E. coli* cytochromes in the mechanism of action of microcin J25. The effect of MccJ25 on different cytochrome mutant strains and membranes isolated from them is addressed. Initially, no significant difference in MICs is observed between wild type C43 and its terminal oxidases mutant strains when treated with MccJ25. As reported for *E. coli* laboratory strains, there is also no decrease in O_2 consumption induced by the peptide [49].

However, Δ*bo*$_3$ and Δ*bd*-I mutants in which the entry of MccJ25 is facilitated by overexpressing FhuA are less sensitive than the wild type (Figure 1 and Figure 2). Besides, the MIC of MccJ25-GA for *E. coli* C43 Δ*bd*-I (pGC01) increased 16-fold relative to the wild type, and *E. coli* C43 Δ*bo*$_3$ (pGC01) became completely resistant. Since MccJ25-GA acts only on the respiratory chain, we can affirm that the effect of the native peptide on the cytochromes mutants is independent of RNA polymerase inhibition. These results allow us to conclude that cytochromes *bo*$_3$ and *bd*-I are involved in the mechanism of action of MccJ25.

Unexpectedly in the Δ*bo*$_3$/Δ*bd*-II and Δ*bd*-I/Δ*bd*-II strains, the original sensitivity to MccJ25 and MccJ25-GA is recovered. Therefore, it is concluded that cytochrome *bd*-II might exert a protective effect against MccJ25 and MccJ25-GA. To the best of our knowledge, there are no reports on ROS clearance activity or the possible induction of an antioxidant defense system by cytochrome *bd*-II. However, it has been reported that terminal oxidases may have associated peroxidase activity. *E. coli* cytochrome *bd*-I was recently reported that displays significant *in vitro* quinol peroxidase activity and enhances bacterial tolerance to oxidative and nitrosative stress conditions [55–57]. Moreover, *Azotobacter vinelandii* mutants lacking cytochrome *bd*-I were hypersensitive

to metal toxicity, and compounds exerting oxidative stress [58]. Additionally, cytochrome *bd*-I has high catalase activity and the expression of the terminal oxidase is induced by external H_2O_2 [59]. Therefore, further studies should be conducted to elucidate the mechanism by which cytochrome *bd*-II protects against the effect of microcin J25.

Microcin J25 inhibits the *E. coli* respiratory chain, the inhibition of O_2 consumption is observed in pGC01-transformed strains, except for Δbo_3 mutant (Figure 3). As discussed below, it is possible that some of the electrons in the presence of MccJ25 are diverted to ROS production, thereby underestimating the inhibition of oxygen consumption. MccJ25 and its derivative MccJ25-GA inhibit the cellular respiration in the same extent indicating that this effect is independent of the action on RNAP (Additional Figure 3) [17].

As previously reported, ROS are involved in the mechanism of action of the peptide [16,60]. This fact was confirmed using ascorbic acid as an external antioxidant agent, which protects against the deleterious effect of the microcin (Figure 8). Unfortunately, we were unable to observe an increment in ROS production when MccJ25 was added to whole cells. We believe that ROS production *in vivo* is not sufficiently high to observe a significant difference between MccJ25 treated cells and control, or the fluorescent probe utilized is not sensitive enough. Higher concentrations of the peptide should be channeled to the plasma membrane for observing a significant effect *in vivo*. Furthermore, one could speculate that detoxifying enzymes in the cytosol might be rapidly quenching ROS production. As a conclusion, the apparent lack of effect in whole cells might be explained by the low levels of ROS produced under our working conditions. It can be hypothesized that the cellular respiration is a secondary mechanism of action that complements the main effect on the RNA polymerase. However, the fact it cannot be assessed *in vivo* does not mean that it is not important. In order to unravel the ROS production at the membranes we had to move to *in vitro*

experiments. Indeed, a great production of ROS is directly induced in terminal oxidases by MccJ25 (~500% increase over control) when the electrons were supplied directly in the form of reduced quinones to isolated membranes (Figure 7). In addition, as shown in Figure 6, superoxide formation in the presence of the peptide was significantly higher in membranes of strains containing cytochrome *bd*-I (wild type, Δ*bo*$_3$, and CBDI). Moreover, in the absence of cytochrome *bd*-I, the overproduction of ROS is very low or not detected (membranes from Δ*bd*-I and CBO strains). Both cytochromes are inhibited, but the way the peptide affects them could be different. The large production of ROS induced *in vitro* in membranes containing cytochrome *bd*-I do not correlate directly with the antibiotic effect observed *in vivo*. In fact, the cytochrome *bd*-I mutant strain would be expected to be much less sensitive than the cytochrome *bo*$_3$ mutant if the overproduction of ROS were the only important factor for anti-bacterial activity. It is plausible to speculate that ROS can affect cells to some extent, thus triggering an unidentified mechanism that in turn would lead to cell death.

In addition, the peptide was not able to inhibit the NADH dehydrogenase activity in membranes when cyanide was present (Additional Figure 4). However, a small inhibition of NADH oxidase activity is observed (Figure 4). As previously explained, this determination is performed in the absence of cyanide. These results suggest that NADH oxidation could be indirectly affected by the inhibition of terminal oxidases located downstream in the respiratory chain. In effect, the inhibition is greater if the determination is carried out using NADH as an electron donor for the respiratory chain and the oxygen consumption is measured (Figure 5). Moreover, in the presence of SOD, catalase and MccJ25, the rate of oxygen consumption falls dramatically. This confirms a simultaneous increased production of toxic oxygen species, with the subsequent release of O_2 by detoxifying enzymes (Figure 5D). KCN dramatically halts oxygen consumption under such conditions (Additional Figure 5). It can be deduced from all these experiments that a flow of electrons to the terminal oxidases is

necessary for MccJ25 to exert its inhibitory action on NADH dehydrogenase activity and ROS overproduction. This is in agreement with previous work done with mitochondria [20].

Therefore, MccJ25 affects the respiratory chain and may act on the target cell in different ways: 1) MccJ25 induce ROS overproduction on cytochrome *bd*-I and inhibits quinol oxidases, producing an accumulation of reduced species upstream of the inhibition site, which increases further the production of ROS [61–63]; 2) Microcin J25 produces an energetic imbalance of the target cell, whereas ROS may affect key enzymes of bacterial metabolism mainly those contain catalytic $[4Fe\text{-}4S]^{2+}$ clusters [64]; 3) Inhibition of RNA synthesis induced by the peptide makes it impossible for the affected cell to elaborate the necessary defense based on the synthesis of new enzymes, whose expression is induced under oxidative stress conditions [61].

5. Conclusions

Both terminal cytochromes *bd*-I and bo_3 demonstrated to be the target on which microcin J25 acts at the level of the respiratory chain, being the cytochrome *bd*-I the major site of superoxide formation induced by the antibiotic peptide. Interestingly, cytochrome *bd*-I plays a crucial role in pathogenic bacteria, becoming a potential drug target for eradicating bacterial infection. The importance of this work is that for the first time a link between terminal oxidases and the mechanism of action of MccJ25 in *E. coli* is established. Unexpectedly, our data suggest that cytochrome *bd*-II protects the target cell from the deleterious effect of MccJ25, possibly through ROS detoxification. A future work will elucidate the role of cytochrome *bd*-II in MccJ25 resistance. Additionally, the mechanism of inhibition of the terminal oxidases by MccJ25 will be studied with the purified proteins. These assays are currently being carried out in our laboratory.

Acknowledgments: The authors want to thank the financial support of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) with grants PIP 0779

and PIP 06906 CO, PICT 2998 from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and PIUNT D548/1 from Universidad Nacional de Tucumán (UNT).

References:

[1] R.A. Salomón, R.N. Farías, Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*, J. Bacteriol. 174 (1992) 7428–7435.

[2] R. Hammami, A. Zouhir, C. Le Lay, J. Ben Hamida, I. Fliss, BACTIBASE second release: a database and tool platform for bacteriocin characterization, BMC Microbiol. 10 (2010) 22. doi:10.1186/1471-2180-10-22.

[3] D. Destoumieux-Garzón, J. Peduzzi, S. Rebuffat, Focus on modified microcins: structural features and mechanisms of action, Biochimie. 84 (2002) 511–519. doi:10.1016/S0300-9084(02)01411-6.

[4] M.J. Bayro, J. Mukhopadhyay, G.V.T. Swapna, J.Y. Huang, L.-C. Ma, E. Sineva, P.E. Dawson, G.T. Montelione, R.H. Ebright, Structure of antibacterial peptide microcin J25: a 21-residue lariat protoknot, J. Am. Chem. Soc. 125 (2003) 12382–12383. doi:10.1021/ja036677e.

[5] K.J. Rosengren, R.J. Clark, N.L. Daly, U. Göransson, A. Jones, D.J. Craik, Microcin J25 has a threaded sidechain-to-backbone ring structure and not a head-to-tail cyclized backbone, J. Am. Chem. Soc. 125 (2003) 12464–12474. doi:10.1021/ja0367703.

[6] K.-A. Wilson, M. Kalkum, J. Ottesen, J. Yuzenkova, B.T. Chait, R. Landick, T. Muir, K. Severinov, S.A. Darst, Structure of microcin J25, a peptide inhibitor of bacterial RNA polymerase, is a lassoed tail, J. Am. Chem. Soc. 125 (2003) 12475–12483. doi:10.1021/ja036756q.

[7] S. Sable, A.M. Pons, S. Gendron-Gaillard, G. Cottenceau, Antibacterial activity evaluation of microcin J25 against diarrheagenic *Escherichia coli*, Appl. Environ. Microbiol. 66 (2000) 4595–4597.

[8] M.F. Pomares, N.S. Corbalán, C. Adler, R. de Cristóbal, R.N. Farías, M.A. Delgado, P.A. Vincent, Macrophage environment turns otherwise MccJ25-resistant *Salmonella* into sensitive, BMC Microbiol. 13 (2013) 95. doi:10.1186/1471-2180-13-95.

[9] R.A. Salomón, R.N. Farías, The FhuA protein is involved in microcin 25 uptake, J. Bacteriol. 175 (1993) 7741–7742.

[10] D. Destoumieux-Garzón, S. Duquesne, J. Peduzzi, C. Goulard, M. Desmadril, L. Letellier, S. Rebuffat, P. Boulanger, The iron-siderophore transporter FhuA is the receptor for the antimicrobial peptide microcin J25: role of the microcin Val11-Pro16 beta-hairpin region in the recognition mechanism, Biochem. J. 389 (2005) 869–876. doi:10.1042/BJ20042107.

[11] R.A. Salomón, R.N. Farías, The peptide antibiotic microcin 25 is imported through the TonB pathway and the SbmA protein, J. Bacteriol. 177 (1995) 3323–3325.

[12] N. Corbalan, G. Runti, C. Adler, S. Covaceuszach, R.C. Ford, D. Lamba, K. Beis, M. Scocchi, P.A. Vincent, Functional and structural study of the dimeric inner membrane protein SbmA, J. Bacteriol. 195 (2013) 5352–5361. doi:10.1128/JB.00824-13.

[13] M.A. Delgado, M.R. Rintoul, R.N. Farías, R.A. Salomón, *Escherichia coli* RNA polymerase is the target of the cyclopeptide antibiotic microcin J25, J. Bacteriol. 183 (2001) 4543–4550. doi:10.1128/JB.183.15.4543-4550.2001.

[14] J. Mukhopadhyay, E. Sineva, J. Knight, R.M. Levy, R.H. Ebright, Antibacterial peptide microcin J25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel, Mol. Cell. 14 (2004) 739–751. doi:10.1016/j.molcel.2004.06.010.

[15] K. Adelman, J. Yuzenkova, A. La Porta, N. Zenkin, J. Lee, J.T. Lis, S. Borukhov, M.D. Wang, K. Severinov, Molecular mechanism of transcription inhibition by peptide antibiotic Microcin J25, Mol. Cell. 14 (2004) 753–762. doi:10.1016/j.molcel.2004.05.017.
[16] A. Bellomio, P.A. Vincent, B.F. de Arcuri, R.N. Farías, R.D. Morero, Microcin J25 has dual and independent mechanisms of action in *Escherichia coli*: RNA polymerase inhibition and increased superoxide production, J. Bacteriol. 189 (2007) 4180–4186. doi:10.1128/JB.00206-07.
[17] A. Bellomio, M.R. Rintoul, R.D. Morero, Chemical modification of microcin J25 with diethylpyrocarbonate and carbodiimide: evidence for essential histidyl and carboxyl residues, Biochem. Biophys. Res. Commun. 303 (2003) 458–462.
[18] M.V. Niklison Chirou, C.J. Minahk, R.D. Morero, Antimitochondrial activity displayed by the antimicrobial peptide microcin J25, Biochem. Biophys. Res. Commun. 317 (2004) 882–886. doi:10.1016/j.bbrc.2004.03.127.
[19] M. Niklison Chirou, A. Bellomio, F. Dupuy, B. Arcuri, C. Minahk, R. Morero, Microcin J25 induces the opening of the mitochondrial transition pore and cytochrome c release through superoxide generation, FEBS J. 275 (2008) 4088–4096. doi:10.1111/j.1742-4658.2008.06550.x.
[20] M.V. Niklison-Chirou, F. Dupuy, L.B. Pena, S.M. Gallego, M.L. Barreiro-Arcos, C. Avila, C. Torres-Bugeau, B.E. Arcuri, A. Bellomio, C. Minahk, R.D. Morero, Microcin J25 triggers cytochrome c release through irreversible damage of mitochondrial proteins and lipids, Int. J. Biochem. Cell Biol. 42 (2010) 273–281. doi:10.1016/j.biocel.2009.11.002.
[21] V.B. Borisov, M.I. Verkhovsky, Oxygen as Acceptor, EcoSal Plus. 6 (2015).
[22] P.A. Cotter, V. Chepuri, R.B. Gennis, R.P. Gunsalus, Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the fnr gene product, J. Bacteriol. 172 (1990) 6333–6338.
[23] V. Chepuri, L. Lemieux, D.C. Au, R.B. Gennis, The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome o ubiquinol oxidase of *Escherichia coli* and the aa3-type family of cytochrome c oxidases, J. Biol. Chem. 265 (1990) 11185–11192.
[24] J. Abramson, S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, M. Wikström, The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site, Nat. Struct. Biol. 7 (2000) 910–917. doi:10.1038/82824.
[25] V.B. Borisov, R.B. Gennis, J. Hemp, M.I. Verkhovsky, The cytochrome *bd* respiratory oxygen reductases, Biochim. Biophys. Acta. 1807 (2011) 1398–1413. doi:10.1016/j.bbabio.2011.06.016.
[26] J. Dassa, H. Fsihi, C. Marck, M. Dion, M. Kieffer-Bontemps, P.L. Boquet, A new oxygen-regulated operon in *Escherichia coli* comprises the genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*), Mol. Gen. Genet. 229 (1991) 341–352.
[27] H.E. Jesse, T.L. Nye, S. McLean, J. Green, B.E. Mann, R.K. Poole, Cytochrome *bd*-I in *Escherichia coli* is less sensitive than cytochromes *bd*-II or *bo* to inhibition by the carbon monoxide-releasing molecule, CORM-3: N-acetylcysteine reduces CO-RM uptake and inhibition of respiration, Biochim. Biophys. Acta. 1834 (2013) 1693–1703. doi:10.1016/j.bbapap.2013.04.019.
[28] B. Miroux, J.E. Walker, Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, J. Mol. Biol. 260 (1996) 289–298. doi:10.1006/jmbi.1996.0399.
[29] L.L. Yap, R.I. Samoilova, R.B. Gennis, S.A. Dikanov, Characterization of mutants that change the hydrogen bonding of the semiquinone radical at the QH site of the cytochrome bo_3 from *Escherichia coli*, J. Biol. Chem. 282 (2007) 8777–8785. doi:10.1074/jbc.M611595200.

[30] J. Hoeser, S. Hong, G. Gehmann, R.B. Gennis, T. Friedrich, Subunit CydX of *Escherichia coli* cytochrome *bd* ubiquinol oxidase is essential for assembly and stability of the di-heme active site, FEBS Lett. 588 (2014) 1537–1541. doi:10.1016/j.febslet.2014.03.036.
[31] M.A. Delgado, R.A. Salomón, Molecular characterization of a DNA fragment carrying the basic replicon of pTUC100, the natural plasmid encoding the peptide antibiotic microcin J25 system, Plasmid. 53 (2005) 258–262. doi:10.1016/j.plasmid.2004.09.003.
[32] J.O. Solbiati, M. Ciaccio, R.N. Farías, R.A. Salomón, Genetic analysis of plasmid determinants for microcin J25 production and immunity, J. Bacteriol. 178 (1996) 3661–3663.
[33] G. Carmel, D. Hellstern, D. Henning, J.W. Coulton, Insertion mutagenesis of the gene encoding the ferrichrome-iron receptor of *Escherichia coli* K-12, J. Bacteriol. 172 (1990) 1861–1869.
[34] M. Bonhivers, A. Ghazi, P. Boulanger, L. Letellier, FhuA, a transporter of the *Escherichia coli* outer membrane, is converted into a channel upon binding of bacteriophage T5, EMBO J. 15 (1996) 1850–1856.
[35] V. Braun, K. Schaller, H. Wolff, A common receptor protein for phage T5 and colicin M in the outer membrane of *Escherichia coli* B, Biochim. Biophys. Acta. 323 (1973) 87–97.
[36] V. Braun, J. Frenz, K. Hantke, K. Schaller, Penetration of colicin M into cells of *Escherichia coli*, J. Bacteriol. 142 (1980) 162–168.
[37] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Pr, 1972.
[38] A. Blond, J. Péduzzi, C. Goulard, M.J. Chiuchiolo, M. Barthélémy, Y. Prigent, R.A. Salomón, R.N. Farías, F. Moreno, S. Rebuffat, The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*, Eur. J. Biochem. 259 (1999) 747–755.
[39] D.G. Hoare, D.E. Koshland, A method for the quantitative modification and estimation of carboxylic acid groups in proteins, J. Biol. Chem. 242 (1967) 2447–2453.
[40] A. Bellomio, R.G. Oliveira, B. Maggio, R.D. Morero, Penetration and interactions of the antimicrobial peptide, microcin J25, into uncharged phospholipid monolayers, J Colloid Interface Sci. 285 (2005) 118–124. doi:10.1016/j.jcis.2004.11.025.
[41] A. Mayr-Harting, A.J. Hedges, R.C.W. Berkeley, Chapter VII Methods for Studying Bacteriocins, in: J.R.N. and D.W. Ribbons (Ed.), Methods in Microbiology, Academic Press, 1972: pp. 315–422.
[42] D.J. Evans Jr, Membrane adenosine triphosphatase of *Escherichia coli*: activation by calcium ion and inhibition by monovalent cations, J. Bacteriol. 100 (1969) 914–922.
[43] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[44] L. Rodríguez-Montelongo, R.N. Farías, E.M. Massa, Sites of electron transfer to membrane-bound copper and hydroperoxide-induced damage in the respiratory chain of *Escherichia coli*, Arch. Biochem. Biophys. 323 (1995) 19–26.
[45] R. Cathcart, E. Schwiers, B.N. Ames, Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay, Anal. Biochem. 134 (1983) 111–116.
[46] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5 (1992) 227–231.
[47] M.C. Bennett, G.W. Mlady, Y.-H. Kwon, G.M. Rose, Chronic *in vivo* sodium azide infusion induces selective and stable inhibition of cytochrome *c* oxidase, Journal

of Neurochemistry. 66 (1996) 2606–2611. doi:10.1046/j.1471-4159.1996.66062606.x.
[48] A.W. Bauer, W.M. Kirby, J.C. Sherris, M. Turck, Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. 45 (1966) 493–496.
[49] M.R. Rintoul, B.F. de Arcuri, R.A. Salomón, R.N. Farías, R.D. Morero, The antibacterial action of microcin J25: evidence for disruption of cytoplasmic membrane energization in *Salmonella newport*, FEMS Microbiol. Lett. 204 (2001) 265–270.
[50] M.G. Bonini, C. Rota, A. Tomasi, R.P. Mason, The oxidation of 2',7'-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy?, Free Radic. Biol. Med. 40 (2006) 968–975. doi:10.1016/j.freeradbiomed.2005.10.042.
[51] H. Culakova, V. Dzugasova, Y. Gbelska, J. Subik, Antibacterial activity of CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine) generating reactive oxygen species, Microbiol. Res. 168 (2013) 147–152. doi:10.1016/j.micres.2012.10.003.
[52] D. Hevia, J.C. Mayo, D.-X. Tan, A. Rodriguez-Garcia, R.M. Sainz, Melatonin enhances photo-oxidation of 2',7'-dichlorodihydrofluorescein by an antioxidant reaction that renders N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), PLoS ONE. 9 (2014) e109257. doi:10.1371/journal.pone.0109257.
[53] M. Ruiz-Leal, S. George, An in vitro procedure for evaluation of early stage oxidative stress in an established fish cell line applied to investigation of PHAH and pesticide toxicity, Mar. Environ. Res. 58 (2004) 631–635. doi:10.1016/j.marenvres.2004.03.054.
[54] M. Goswami, S.H. Mangoli, N. Jawali, Involvement of reactive oxygen species in the action of ciprofloxacin against *Escherichia coli*, Antimicrob. Agents Chemother. 50 (2006) 949–954. doi:10.1128/AAC.50.3.949-954.2006.
[55] S. Al-Attar, Y. Yu, M. Pinkse, J. Hoeser, T. Friedrich, D. Bald, S. de Vries, Cytochrome *bd* displays significant quinol peroxidase activity, Sci Rep. 6 (2016) 27631. doi:10.1038/srep27631.
[56] A. Giuffrè, V.B. Borisov, M. Arese, P. Sarti, E. Forte, Cytochrome *bd* oxidase and bacterial tolerance to oxidative and nitrosative stress, Biochim. Biophys. Acta. 1837 (2014) 1178–1187. doi:10.1016/j.bbabio.2014.01.016.
[57] V.B. Borisov, E. Forte, S.A. Siletsky, P. Sarti, A. Giuffrè, Cytochrome *bd* from *Escherichia coli* catalyzes peroxynitrite decomposition, Biochim. Biophys. Acta. 1847 (2015) 182–188. doi:10.1016/j.bbabio.2014.10.006.
[58] S.E. Edwards, C.S. Loder, G. Wu, H. Corker, B.W. Bainbridge, S. Hill, R.K. Poole, Mutation of cytochrome *bd* quinol oxidase results in reduced stationary phase survival, iron deprivation, metal toxicity and oxidative stress in *Azotobacter vinelandii*, FEMS Microbiol. Lett. 185 (2000) 71–77. doi:10.1111/j.1574-6968.2000.tb09042.x.
[59] V.B. Borisov, E. Forte, A. Davletshin, D. Mastronicola, P. Sarti, A. Giuffrè, Cytochrome *bd* oxidase from *Escherichia coli* displays high catalase activity: an additional defense against oxidative stress, FEBS Lett. 587 (2013) 2214–2218. doi:10.1016/j.febslet.2013.05.047.
[60] M.C. Chalon, A. Bellomio, J.O. Solbiati, R.D. Morero, R.N. Farias, P.A. Vincent, Tyrosine 9 is the key amino acid in microcin J25 superoxide overproduction, FEMS Microbiol. Lett. 300 (2009) 90–96. doi:10.1111/j.1574-6968.2009.01770.x.
[61] J.A. Imlay, The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium, Nat. Rev. Microbiol. 11 (2013) 443–454. doi:10.1038/nrmicro3032.
[62] K.R. Messner, J.A. Imlay, The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*, J. Biol. Chem. 274 (1999) 10119–10128.
[63] K.R. Messner, J.A. Imlay, Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase, J. Biol. Chem. 277 (2002) 42563–42571. doi:10.1074/jbc.M204958200.

[64] S. Varghese, Y. Tang, J.A. Imlay, Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion, J. Bacteriol. 185 (2003) 221–230.

Figure Legends

Fig 1. Sensitivity by spot-on-lawn test. Minimal inhibitory concentrations (MIC) of MccJ25 and its chemical derivative MccJ25-GA against different strains of *E. coli* C43. CBO strain is completely resistant to MccJ25 because it has a *fhuA* mutation. It turns into sensitive after pGC01 transformation.

Fig 2. Cell viability. Cells from the different strains of *E. coli* C43 (pGC01) were grown to OD_{600} of 0.8. The cultures were divided into two portions, and one of them was treated with 20 µM MccJ25; the other was left as a control. The white bars represent the controls of each strain without peptide. Data are expressed as mean ± SD. Student's t-test was used to determine the statistical significance; no asterisk: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$ and ****: $P \leq 0.0001$. Three independent experiments were performed.

Fig 3. Effect of MccJ25 on oxygen consumption. *E. coli* C43 (pGC01) cells were grown to exponential phase (OD_{600} 0.8) in LB medium. Samples were diluted to an OD_{600} of 0.15 and incubated at 37°C for 30 minutes with and without MccJ25. The inhibition percentages are related to a control without MccJ25. Data are expressed as mean ± SD. One way ANOVA was used to determine the statistical significance; no asterisk: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$ and ***: $P \leq 0.001$. Three independent experiments were performed.

Fig 4. Effect of MccJ25 on NADH oxidase activity. The activity was followed by measuring NADH consumption at 340 nm using *E. coli* C43 membranes (10 µg/ml protein). The membranes were pre-incubated for 15 minutes with or without MccJ25 20 µM. Data are expressed as mean ± SD. Two way ANOVA was used to determine the statistical significance; no asterisk: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$ and ***: $P \leq 0.001$. Three independent experiments were performed.

Fig 5. Effect of SOD and catalase on NADH oxidase activity: The oxygen consumption was measured using isolated membranes from *E. coli* C43 wild type strain in the presence of 10 mM NADH (added after 10 min preincubation of membranes with 20 µM MccJ25). The reaction was performed with and without 150 IU Mn-SOD. The control contained methanol instead of MccJ25. Bovine liver catalase (425 IU) was added at ~ 20 minutes of reaction. The oxygen concentration is represented as a function of time from minute 15 (after 10 minutes of preincubation and 5 minutes for system stabilization). The graph is representative of three experiments in which slopes values are obtainedThe average values of the slopes (pmol . s^{-1} . ml^{-1}) are shown in the figure..The rate of oxygen consumption was calculated as the first derivative of the concentration of O_2 with respect to time using Graph Pad Software (first derivative with 2^{nd} order smoothing (4 neighbors)).

Fig 6. Superoxide overproduction induced by MccJ25. *E. coli* C43 membranes were incubated with 3 µM DCFH and 20 µM MccJ25 or methanol (control). The reaction was initiated with 10 mM sodium succinate. The DCF fluorescence (λ_{exc} 490 nm - λ_{em} 520 nm) was measured after 30 minutes. The values show the difference between DCFH oxidation without and with SOD (10 IU). Data are expressed as mean ± SD. Two way ANOVA was used to determine the statistical significance; no asterisk: P > 0.05, *: P ≤ 0.05, **: P ≤ 0.01 and ***: P ≤ 0.001 and ****: P ≤ 0.0001. Three independent experiments were performed.

Figure 7. ROS production with reduced quinone. *E. coli* C43 WT membranes were incubated with 3 µM DCFH and 20 µM MccJ25 or methanol (control). The reaction was initiated with 100 µM QH_2. The DCF fluorescence (λ_{exc} 490 nm - λ_{em} 520 nm) was measured during 30 minutes. Data are expressed as mean ± SD. The data shown are representative of at least three separate studies performed in triplicate.

Fig 8. Antimicrobial activity of MccJ25 in the presence of an antioxidant agent. Decreased sensitivity of different *E. coli* C43 strains against MccJ25 in the presence of

5 mM ascorbic acid. Ten microliters of each MccJ25 concentration were spotted on the disk.

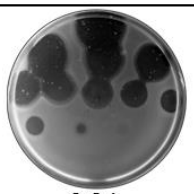
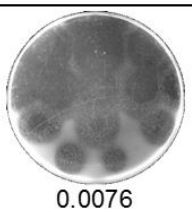
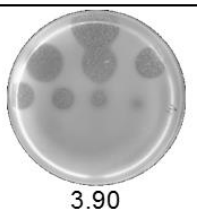
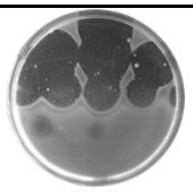
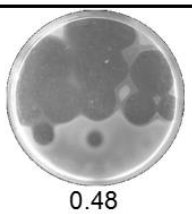
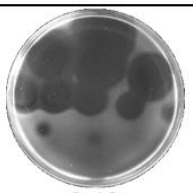
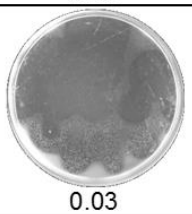
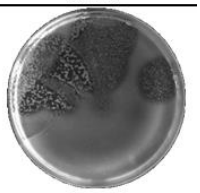
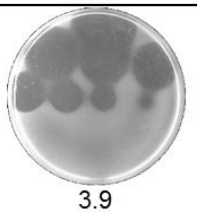
Strain	Cytochrome present	MIC (μM)			
		MccJ25		MccJ25 - GA	
			+ pGC01		+ pGC01
WT	*bo*$_3$ *bd*-I *bd*-II	 0.24	 0.0076	 125	 3.90
Δ*bo*$_3$	*bd*-I *bd*-II	 0.24	 0.48	 > 500	 > 500
Δ*bd*-I	*bo*$_3$ *bd*-II	 0.48	 0.48	 125	 62.5
CBDI	*bd*-I	 0.48	 0.03	 125	 3.90
CBO	*bo*$_3$	 > 500	 0.015	 > 500	 3.9

Fig. 1

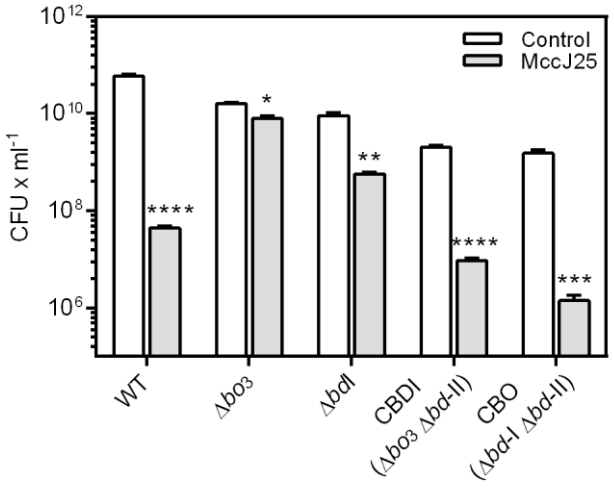


Fig. 2

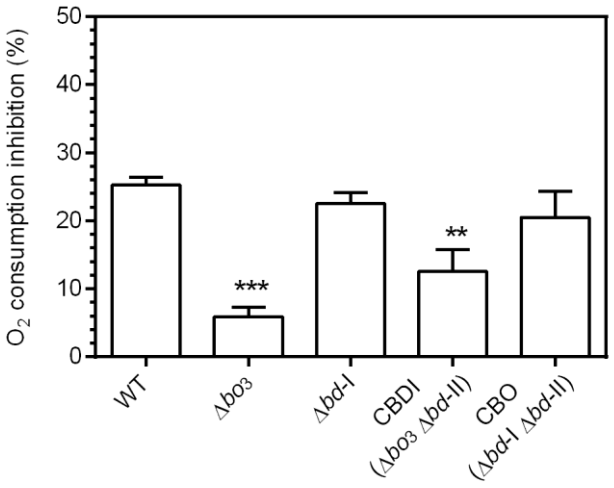


Fig. 3

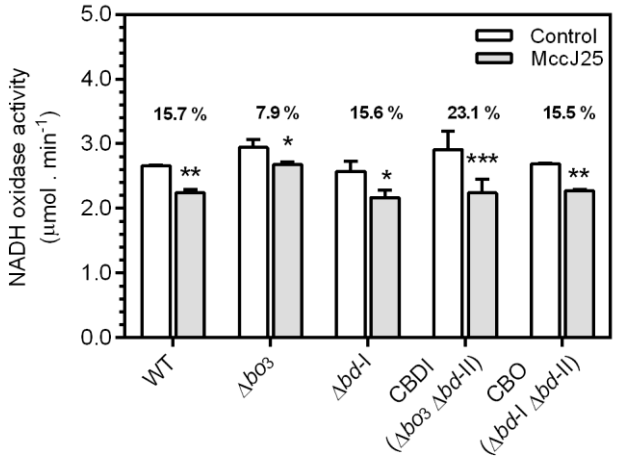


Fig. 4

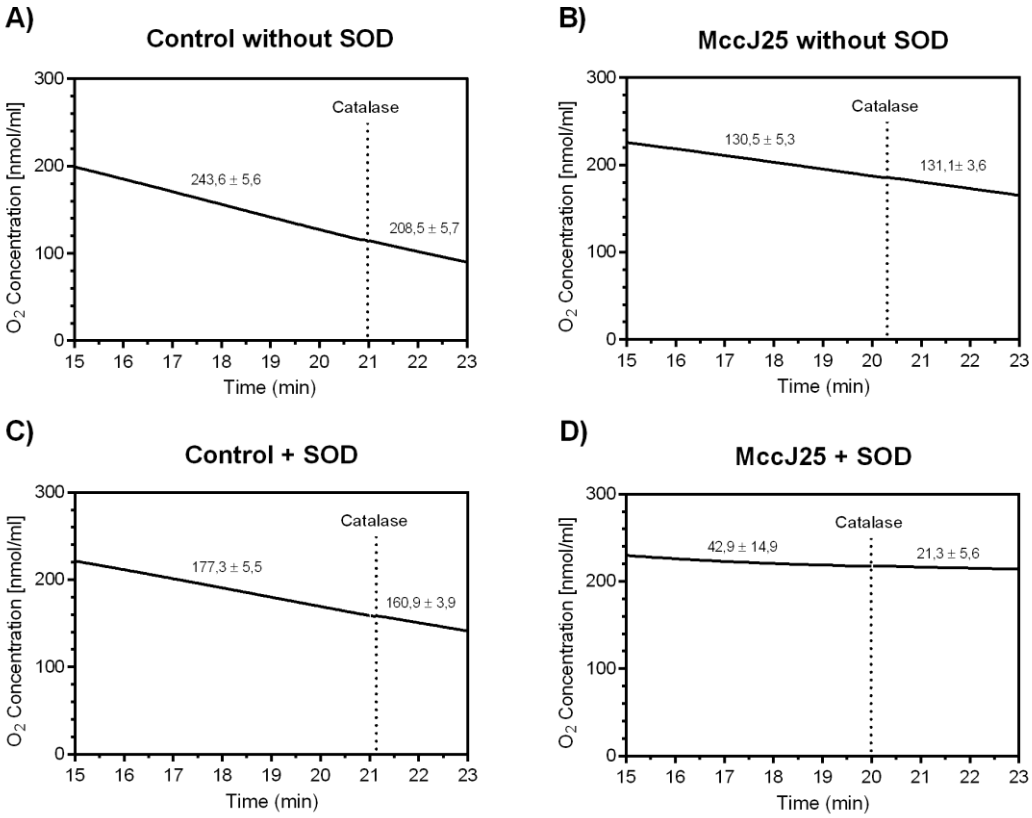


Fig. 5

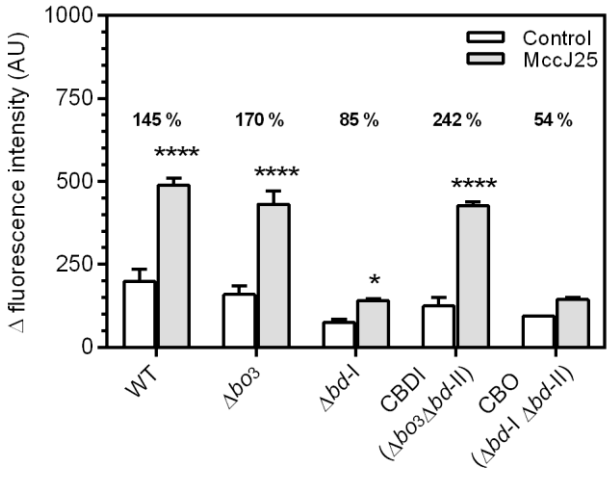


Fig. 6

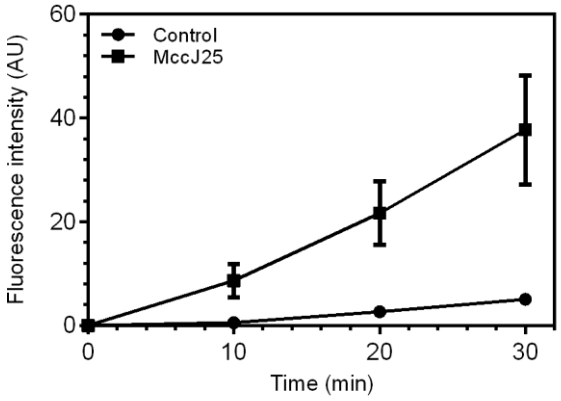


Fig. 7

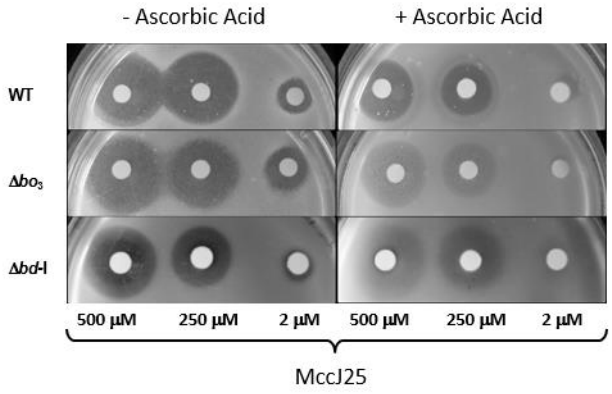
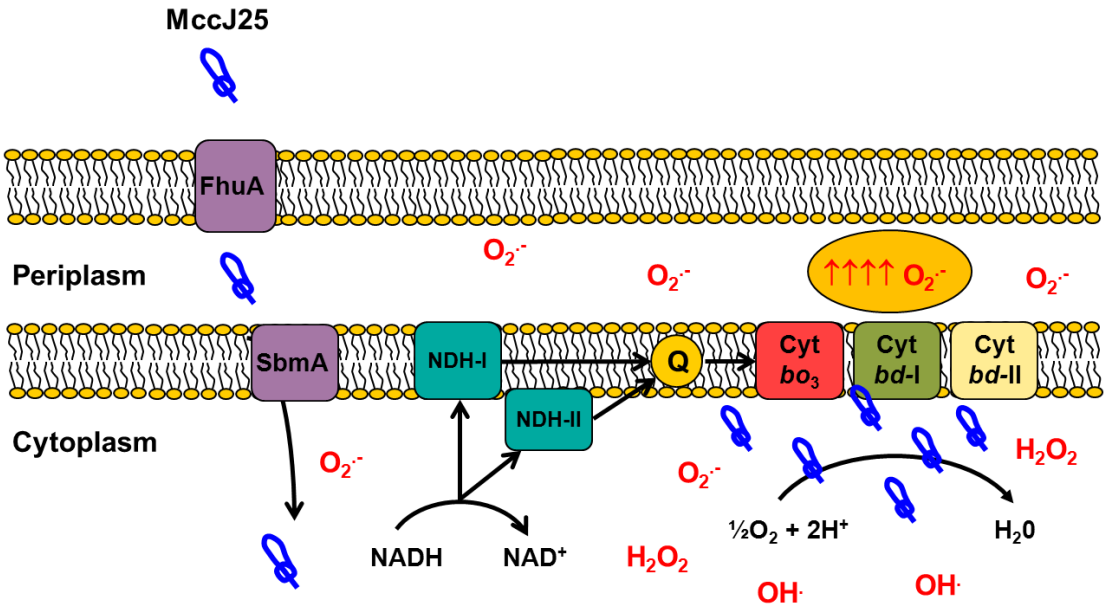


Fig. 8



Graphical abstract

Highlights

- *E. coli* cytochromes *bd*-I and bo_3 are involved in the mechanism of action of MccJ25
- Cytochrome *bd*-I would be the main site of superoxide formation induce by MccJ25
- MccJ25 is the only known microcin that acts on the respiratory chain of *E. coli*