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New insights into the mechanism of antibacterial action of synthetic peptide *Mo*-CBP$_3$-PepI against *Klebsiella pneumoniae*

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Abstract: *Klebsiella pneumoniae* is a multidrug-resistant opportunistic human pathogen related to various infections. As such, synthetic peptides have emerged as potential alternative molecules. *Mo*-CBP$_3$-PepI has presented great activity against *K. pneumoniae* by presenting an MIC$_{50}$ at a very low concentration (31.25 μg mL^{-1}). Here, fluorescence microscopy and proteomic analysis revealed the alteration in cell membrane permeability, ROS overproduction, and protein profile of *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI. *Mo*-CBP$_3$-PepI led to ROS overaccumulation and membrane pore formation in *K. pneumoniae* cells. Furthermore, the proteomic analysis highlighted changes in essential metabolic pathways. For example, after treatment of *K. pneumoniae* cells with *Mo*-CBP$_3$-PepI, it was seen a reduction in the abundance of protein related to DNA and protein metabolism, cytoskeleton and cell wall organization, redox metabolism, regulation factors, ribosomal proteins, and resistance to antibiotics. These reductions lead to the inhibition of DNA repair, inhibition of cell wall turnover, protein turnover, and ROS accumulation leading to cell death. Our findings indicated that *Mo*-CBP$_3$-PepI might have mechanisms of action against *K. pneumoniae* cells, mitigating the development of resistance and thus being a potent molecule to be employed in producing new drugs against *K. pneumoniae* infections.

Keywords: Antimicrobial peptides; Synthetic peptides; multidrug resistant bacteria; proteomic analysis

1. Introduction

The emergence of multidrug-resistant bacteria (MDRB) is a challenge to public health worldwide, leading to lengthy of stay in hospitals, high costs for health care, and deaths [1–3]. Among those MDRBs is *Klebsiella pneumoniae*, a gram-negative normally found in the human gastrointestinal system and can also be found in feces [4]. Additionally, *K. pneumoniae* is a bacterium belonging to the ESKAPE group. The ESKAPE group is composed of six MDRB pathogenic to humans as follows *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *K. pneumonia* [5,6].

K. pneumoniae is an opportunistic MDRB only able to infect people with a compromised immune system [4]. *K. pneumoniae* presents resistance against Polymyxins, Carbapenems, fluoroquinolones, aminoglycosides, tetracyclines, third-generation cephalosporins, and pan drug-resistant [7–9]. The clinical manifestations of *K. pneumoniae* as acute bacterial skin infection, bacteremia, pneumonia, and osteoarticular infection [10–12].

Now is clear the importance of *K. pneumonia* to human health and the problems with the antidrug resistance developed by it. Based on that, it is emergent and imperative to seek new molecules to fight back in two ways: 1) to develop a new drug to produce a new treatment; or 2) to produce a new molecule that could act synergistically with commercial

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drugs making them effective again [1]. In this scenario, antimicrobial peptides represent a notorious group of molecules that could help scientists to find quickly a way to cope with antimicrobial resistance [13]. However, natural antimicrobial peptides have presented problems in clinical trials, such as toxicity to host cells, low resistance to proteolysis, and sometimes high cost of obtention [13,14].

To solve the problems presented by natural antimicrobial peptides, synthetic antimicrobial peptides emerged as a solution. Synthetic antimicrobial peptides are designed using a natural model sequence to have higher antimicrobial activity, resistance to proteolysis, and no toxicity to host cells. Recently, our research group has designed synthetic peptides called *Mo*-CBP$_3$-PepI using the sequence of a chitin-binding protein from *Moringa oleifera* [15]. *Mo*-CBP$_3$-PepI is a non-hemolytic small cationic peptide with a net charge +1, a molecular mass of 893.10 Da, and a hydrophobic ratio of 62%. It has a secondary structure as α-helix confirmed by circular dichroism assays [15].

Regarding the antimicrobial potential, *Mo*-CBP$_3$-PepI presents great anticandidal activity against *Candida albicans*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*. The mechanisms of action behind the anticandidal activity of *Mo*-CBP$_3$-PepI were revealed using *C. albicans* as a model [15,16]. The antibacterial activity of *Mo*-CBP$_3$-PepI was only relevant against *K. pneumoniae*. Therefore, this study employed fluorescence and scanning electron microscopes and proteomic analysis to provide new insights into the mechanism of antibacterial action of *Mo*-CBP$_3$-PepI against *K. pneumoniae*. Additionally, it was toxicity tests against human cells to produce new data on the safety of *Mo*-CBP$_3$-PepI.

2. Material and methods

2.1. Biological material

The human-pathogenic Gram-negative bacteria *K. pneumoniae* (ATCC 10031) strain was obtained from the Laboratory of Plant Toxins (LABTOX) of the Federal University of Ceará (UFC).

2.2. Peptide synthesis

The synthetic peptide *Mo*-CBP$_3$-PepI (CPAIQRCC) [15] was chemically synthesized by the company ChemPeptide (Shanghai, China), where its purity and quality of it were tested by mass spectrometry and reverse-phase high-performance liquid chromatography.

2.3. Cell viability by MTT assay

The cell viability assay was performed as described by Lima et al. [17], with some adjustments. After the antibacterial assay was done as described by Oliveira et al. [15], the wells containing the treated cells and control cells (50 µL) were incubated for 3h in the dark at 37 ºC with 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2 mg mL^{-1}, MTT). After incubation, 100 µL of 100% DMSO was added to the wells, and the plate was slowly shaken to dissolve the formazan crystals. The absorbance was measured using a microplate reader (Epoch, BioTek) with a wavelength of 495 nm. The controls used for this assay were 5% of dimethylsulfoxide (DMSO) prepared in 0.15M NaCl (saline) solution and ciprofloxacin (1000 µg mL^{-1}) prepared in 5% ethanol in sterile saline solution.

2.4. Antibiofilm assay

The antibiofilm assay was conducted in flat-bottom 96-well polystyrene microplates as described by Neto et al. [18]. A single colony of *K. pneumoniae* was collected in stock Petri plates containing inoculated Mueller Hinton Agar. The colony was used as inoculum for 5 mL of Mueller Hinton broth and the media was incubated for 24 h in the dark at 37 ºC. In sequence, the O.D. of the cell suspension was measured using a microplate reader (Epoch, BioTek) and adjusted to 0.1 at 630 nm with Mueller Hinton broth. For the inhibition of biofilm formation, 50 µL of the cell suspension was incubated with 50 µL of the peptide solution and 50 µL of the respective controls described above. The incubation

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lasted for 48 h at 37 ºC. For the biofilm degradation assay, 50 µL of the cell suspension was incubated for 24 h at 37 ºC. After this, 50 µL of the peptide solution and the respective controls were added to the wells containing the preformed biofilm and incubated for 24 h at 37 ºC.

After incubation, both wells of inhibition of biofilm formation and biofilm degradation were washed once with a sterile saline solution. Then, the wells were fixated with 100 µL methanol 99% for 15 min. After the methanol was removed and the plates were dried at 37 ºC. Then, the wells were stained with 200 µL of 0.1% violet crystal solution for 20 min. In sequence, the wells were washed three times with distilled water. Then, the crystals stained on the biofilm were diluted with 200 µL of 33% acetic acid, and the O.D was measured using a wavelength of 600 nm. The assay was made in triplicate, with three independent biological experiments.

2.5. Mechanism of action evaluation by fluorescence microscopy

2.5.1. Cell membrane integrity by propidium iodide (PI) and FITC-Dextran uptake

The assay was conducted as described by Oliveira et al. [15], with some modifications. Preparation of cell suspension was done as mentioned above. After, 50 µL of the diluted bacteria solution was incubated in the dark for 24 h, at 37 ºC, with 50 µL of the peptide solution (31.25 µg mL^{-1}) previously prepared with 5% of dimethylsulfoxide (DMSO) diluted in 0.15 M NaCl solution. The assay was conducted in 1.5mL microtubes. The diluted bacteria solution was incubated only with the DMSO-NaCl solution for control. After incubation, the microtubes were centrifuged (5000 g, 5 min, 4 ºC) and washed three times. Next, the washed cells were incubated with 10^3 µM propidium iodide for 30 min, in the dark, at 37 ºC. After this, the cells were washed two times with saline solution to remove the excess fluorophore and observed with a fluorescence microscope (Olympus System BX 60; excitation wavelength, 488 nm; emission wavelength, 525 nm).

Additionally, to know the size of the pore formed, in a new experiment precisely as above, cells were incubated with a FITC-Dextran (fluorescein isothiocyanate (FITC)-Dextran) with a size of 10-kDa. After incubation for 30 min, cells were washed as above and visualized in Olympus System BX 60; excitation wavelength, 490 nm; emission wavelength, 520 nm.

2.5.2. Detection of peptide-induced overproduction of reactive oxygen species (ROS)

This assay was conducted the same as above but with a few differences. The preparation of cell suspension and the antimicrobial assay was done in the same way described above. After the three rounds of centrifugation and washes, the cells were incubated with 1 mM 2, 7 – dichlorofluorescein diacetate (DCFH-DA) [19] for 30 min, in the dark, at 37 ºC. After the washes, the cells were also observed under a fluorescence microscope (Olympus System BX60; excitation wavelength, 488 nm; emission wavelength, 525 nm).

2.4.3. Scanning electronic microscopy (SEM) Analysis

SEM analysis was conducted as described by Staniszewska et al. [20]. After the antibacterial assay, cells were centrifuged (5000 g, 5 min, 4 ºC), the supernatant was removed, cells resuspended, and fixated for 5 h in a fixation solution (2.5% glutaraldehyde [v/v] in 0.15 M Na-phosphate buffer, pH: 7.2). After centrifugations as described above, the cells were washed three times with 0.15 M Na-phosphate buffer, pH: 7.2. For dehydration, the samples were incubated and dried with ethanol (30%, 50%, 70%, 100%, 100% [v/v]) for 10 min each, and centrifugation as described above after each incubation time. Lastly, the samples were incubated with 50/50 ethanol/hexamethyldisilazane (HMDS) for 10 min and centrifuged. Then the pellet was washed with 100% HMDS and transferred to a coverslip to dry out. After complete drying, the coverslips were assembled on stubs and coated with a 20 nm gold layer using a PET coating machine (EMITECH - Q150TES, Quorum Technologies, England). The SEM analyses were done with an Inspect™ 50 FEI Scanning Electron Microscope (Oregon USA), equipped with a low energy detector (EverhartThornley

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detector), using an acceleration beam voltage of 20000 kV and 20000x detector magnification.

2.5. Protein extraction and Gel-Free Proteomic analysis

Initially, an antibacterial assay was performed within 24 h of incubation, using the best inhibitory concentration of *Mo*-CBP$_3$-PepI [15]. After this, samples were washed twice with 50 mM sodium acetate pH 5.2, with centrifugations at 12000 g for 15 minutes at 4 °C. At the end of the washes, the samples were resuspended in 300 µL in the same buffer and frozen for 24 hours. Then the frozen samples were sonicated for 30 minutes to break the cell wall and membrane, the samples were centrifuged again, and the supernatant was collected.

After that, the Bradford assay was performed to determine the protein concentration in the samples. This step was followed by adding a 10 mM DTT solution under incubation for 1 hour at 37 °C to reduce the proteins. Then iodoacetamide was added to a final concentration of 15 mM and incubated for 30 minutes in a dark room to alkylate the reduced proteins. The proteins reduced and alkylated were digested using trypsin gold (Promega, USA) to a final concentration of 1:20 (w/w) as described by manufacturers. The trypsin digestion was performed for 16 hours at 37 °C. Finally, the samples were dried in a speed vacuum (Eppendorf, Germany) for 3 hours and analyzed by ESI-QUAD- TOF mass spectrometer.

2.6. Protein identification

Tandem mass spectra were extracted into PKL files for both samples, and the proteins were searched using MASCOT MS/MS ions search from MATRIX SCIENCE (https://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) against UP625_E_coli_K12 (AA), UP808_K_pneumoniae and SwissProt databases (the taxonomy was set in bacteria). The for the search were: fixed modifications to Carbamidoethyl (C); variable modifications to Oxidation (O); the Peptide tolerance was set to 1.2 DA (with 1% FDR); the MS/MS tolerance was set to 0.6 DA; the peptide charge was set to 2+, 3+ and 4+; and finally the instrument was set to ESI-QUAD-TOF. The proteins identified in both samples were searched for in UNIPROT and separated into 3 sets (unique from control, unique from cells treated with *Mo*-CBP$_3$-PepI, and *Mo*-CBP$_3$-PepI x control shared proteins).

The proteins with a fold-change value ≥ 1.5 ($p<0.05$, Tukey's test) were up-accumulated (increased the abundance), and proteins with a fold-change value ≤ 0.5 ($p<0.05$, Tukey's test) were down-accumulated (decreased the abundance) were taken into consideration for comparisons. For each protein, its corresponding FASTA file was downloaded. Then, the blast2go program (https://www.blast2go.com/) was used to categorize the proteins detected by Gene Ontology (GO) annotation according to Molecular function, Biological Activity, and subcellular location.

3. Results and Discussion

3.1. Cell viability and antibiofilm activity of Mo-CBP3-PepI against K. pneumoniae

As reported in a previous work by Oliveira et al. [15], *Mo*-CBP$_3$-PepI presented an MIC$_{50}$ against *K. pneumoniae* at 31.25 µg mL^{-1}. The experiments we repeated here led to the same results, corroborating the data presented by Oliveira et al. [15]. The concentration of *Mo*-CBP$_3$-PepI to reach an MIC$_{50}$ against *K. pneumoniae* is very low than other synthetic peptides [21,22]. For example, Fleeman et al. [21] showed that the synthetic peptide called PepC, which presented an MIC$_{50}$ at a concentration of 350 µg mL^{-1} 11 times higher than the concentration presented by *Mo*-CBP$_3$-PepI. Additionally, Tincho et al. [22] tested 3 synthetic peptides, and all presented an MIC$_{50}$ against *K. pneumoniae* at a concentration of 500 µg mL^{-1}, 16.07 times higher than *Mo*-CBP$_3$-PepI. These results revealed that *Mo*-CBP$_3$-PepI is much more effective against *K. pneumoniae* than other synthetic peptides.

We further evaluated the cell viability of *K. pneumoniae* cells and biofilm formation after treatment with *Mo*-CBP$_3$-PepI. The first was to evaluate the number of viable cells

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after treatment with *Mo*-CBP$_3$-PepI (Table 1). The MTT assay revealed that only 47.54% ± 0.008 of *K. pneumoniae* cells were viable after incubation with *Mo*-CBP$_3$-PepI, which agrees with the data of MIC$_{50}$. These results prove that *Mo*-CBP$_3$-PepI kills half of the cells at the tested concentration. Otherwise, 100% of *K. pneumoniae* cells were viable in control treated with 5% DMSO in 0.15 M NaCl (Table 1).

Moving forward, the antibiofilm potential of *Mo*-CBP$_3$-PepI toward *K. pneumoniae* was evaluated. *Mo*-CBP$_3$-PepI barely inhibited the biofilm formation of *K. pneumoniae*, only 11.87% ± 0.001, and presented no activity against the preformed biofilm (Table 1). As expected, the control solution was ineffective in inhibiting the formation or degrade preformed biofilms of *K. pneumoniae* (Table 1).

3.2. Toxicity of Mo-CBP$_3$-PepI to human cells

Before we move forward with the study to understand the mechanisms of action of *Mo*-CBP$_3$-PepI against *K. pneumoniae*, toxicity tests against human cells were needed to provide information that might indicate the application of *Mo*-CBP$_3$-PepI. For example, Oliveira et al. [15] presented a safe *Mo*-CBP$_3$-PepI based on the absence of hemolytic activity against human red blood cells even at concentrations (120 μg mL^{-1}) 4 times higher than MIC$_{50}$ concentration [15].

In this study, we went further in the analysis of toxicity. *Mo*-CBP$_3$-PepI was assayed against other human cells (Fig. 1). MTT assay and morphological analysis of human fetal lung fibroblast (MRC-5 line), human keratinocytes (HaCaT line), and L929 fibroblast cells from mice revealed that *Mo*-CBP$_3$-PepI did not affect either cell viability and morphology of those cells even at a concentration of 1 mg mL^{-1}, which is 32 times higher than MIC$_{50}$ concentration. In contrast, the positive control for damage methyl methanesulfonate (MMS) (4×10^{-5} M) led to the death of all cells. It caused severe damage to DNA and nuclei structure indicating the establishment of cell death (Fig. 1).

Another experiment to evaluate damage and fragmentation of DNA caused by *Mo*-CBP$_3$-PepI was evaluated by Cometa assay (Fig. 2) against the same cell lines and at the same concentration. The assay revealed that the DNA of cells treated with peptides presented no damage at all. In contrast, cells treated with MMS presented a DNA completely damaged (Fig. 2). These results assure that the peptide is either safe or presents a very low risk for human cells. Other peptides have presented high toxicity to human red blood cells and other human-type cells, such as WRL-68 (liver cells) and NL-20 (lung cells) [23].

The synthetic peptides RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6, derived from indolicidin and ranalexin, presented 50% of hemolytic activity, respectively, at 62.5, 62.5, 125, and 125 μg mL^{-1} [23]. Additionally, the authors revealed that all peptides RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, RN7-IN6 presented toxicity to WRL-68 (liver cells) NL-20 (lung cells) at concentration of 125 μg mL^{-1} [23]. These concentrations show that those peptides are more toxic than *Mo*-CBP$_3$-PepI. Therefore, based on the results of the toxicity of *Mo*-CBP$_3$-PepI, we decided to move forward in the experiment of the mechanism of action.

3.2. mechanism of action of Mo-CBP$_3$-PepI against K. pneumoniae

3.2.1. membrane pore formation and ROS overproduction

The mechanisms employed by *Mo*-CBP$_3$-PepI against *K. pneumoniae* were evaluated against planktonic lifestyle, given that biofilm activity was not satisfactory (Table 1). The assay to evaluate the pore in the membrane by PI uptake revealed that the treatment with *Mo*-CBP$_3$-PepI induced the pore formation in the membrane of *K. pneumoniae* cells, as revealed by red fluorescence detected (Fig. 3A-B and E-F). The green fluorescence of Dextran-FITC (Fig. 3C-D and G-H) indicated that the pore formed by *Mo*-CBP$_3$-PepI in the membrane of *K. pneumoniae* cells is at least 10 kDa because the size fluorophore used is 10 kDa.

Usually, membranes are the target of peptides, either synthetic or natural [16,24]. Different from antibiotics that generally affect a protein, which lead to a rapid development of resistance, targeting membrane *Mo*-CBP$_3$-PepI imposes a hard problem for *K.*

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pneumoniae cell to cope with. Membranes are a complex structure, highly conserved during cell evolution. So, remodeling plasma membrane upon external stress is metabolically expensive and dangerous to cells [14].

Mo-CBP$_3$-PepI, *Mo*-CBP$_3$-PepII, and *Mo*-CBP$_3$-PepIII are synthetic peptides derived from a chitin-binding protein purified from *Moringa oleifera* [15]. Likewise, *Mo*-CBP$_3$-PepII and *Mo*-CBP$_3$-PepIII, *Mo*-CBP$_3$-PepI to chitin and induce membrane pore in important human pathogenic fungi such as *Candida* spp., *Cryptococcus neoformans*, and *Trichophyton mentagrophytes* [16,17,25]. Here, the bacterial potential and mechanisms of action behind the antibacterial activity of Mo-CBP3-PepI against *K. pneumoniae* cells are presented for the first time. The movement of PI (Fig. 3A-B and E-F) through the membrane of *K. pneumoniae* revealed by red fluorescence indicates the permeabilization by establishing a pore size of 692.50 Da [26,27]. Additionally, the size is estimated to be 10 kDa (Fig. 3C-D and G-H), given the green fluorescence released by Dextran-FITC.

The questions are, how does *Mo*-CBP$_3$-PepI induce pore formation in the membrane of *K. pneumoniae* cells, and why is the pore so big? To answer the first question is necessary to drawback to the design process of *Mo*-CBP$_3$-PepI. During the design process, the *Mo*-CBP$_3$-PepI sequence was achieved to reach three essential features for antimicrobial activity, positive net charge (+1), hydrophobic potential (62%), and probability of 100% to produce a secondary structure in α-helix [13,15,28]. First, the positive net charge of *Mo*-CBP$_3$-PepI given by the presence of an arginine residue is essential for the ionic attraction of *Mo*-CBP$_3$-PepI to negatively charged peptides in the outlier of *K. pneumoniae* membrane and initial insertion on it [29]. Second, the hydrophobic potential critical for interaction with the hydrophobic core of the membrane's lipid bilayer is conferred by the presence of apolar amino acid residues [30]. Third, a secondary structure in α-helix is important to the attraction and insertion of *Mo*-CBP$_3$-PepI into the *K. pneumoniae* [28]. Bioinformatics analysis revealed that *Mo*-CBP$_3$-PepI is a cell-penetrating peptide [15]. It is important to notice that *K. pneumoniae* is a gram-negative bacterium with an outer membrane completely exposed to *Mo*-CBP$_3$-PepI attack by the above mechanism.

To answer the second question is necessary to understand an important characteristic of antimicrobial peptides, the self-association [31]. Self-association is the ability of antimicrobial peptides to interact during the insertion on the membrane, allowing the formation of a huge pore [31]. Based on this result, it is hypothesized the establishment of a barrel-stavel model induces pore formation. In this model, a peptide interacts with lipids in the membrane as described above to perform the insertion into the membrane. Then, peptide molecules interact between them to form a huge pore on the membrane [31–33]. Lima et al. [16] showed that *Mo*-CBP$_3$-PepI induces the formation of a pore of 10 kDa in *C. albicans* cells.

In the same way, the evaluation of ROS overproduction in *K. pneumoniae* cells was evaluated. The treatment of *K. pneumoniae* cells with *Mo*-CBP$_3$-PepI led to a slight accumulation of ROS within cells. In contrast, control cells treated with DMSO did not present any ROS production, which was an expected result (Fig. 1). ROS are essential to cell development. However, the line between good and bad is very tight. The induction of overproduction of ROS could be lethal to cells (Fig 3). The undesired and uncontrolled ROS production and accumulation are associated with damage in important cell life molecules such as proteins, lipids, and DNA, driving cells to death [26]. It is known that *Mo*-CBP$_3$-PepI can induce ROS overproduction in *C. albicans* cells [16]. But, the ability to induce ROS in bacterial cells is revealed for the first time. Rowe-Magnus et al. [34] showed that synthetic Cathelicidin-derived peptides induced ROS overproduction in the gram-negative *Vibrio cholera.* The authors discuss that induction of ROS accumulation was mediated by damage caused in the membrane. Here, we showed that *Mo*-CBP$_3$-PepI could also induce pore formation in the membrane of *K. pneumoniae*, in addition, to ROS overproduction. Based on that, it is possible to correlate the ROS overproduction induced by *Mo*-CBP$_3$-PepI in *K. pneumoniae* cells with the pore formed.

3.2.2. Scanning Electron Microscopy (SEM)

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SEM analysis was employed to provide more insights into the effect of *Mo*-CBP$_3$-PepI on *K. pneumoniae* morphology (Fig. 4). As expected, control *K. pneumoniae* cells treated with DMSO presented a healthy morphology with no damage on the surface (Fig. 4A). However, the *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI several different and lethal damages on the surface (Fig. 4B-H). After treatment with *Mo*-CBP$_3$-PepI, *K. pneumoniae* cells presented cells completely broken with damages on the cell wall, such as depression, abnormal morphology, roughness, and irregular cell surface (Fig. 4B and C – white arrows), and in many cases is possible to see the extravasation of cytoplasmic content may occur due to the pores formed in the membrane (Fig. 4D-F and H – white arrows). Interestingly, *Mo*-CBP$_3$-PepI-treated *K. pneumoniae* cells presented structure-like depressions, broken cell walls, and contorted cells (Fig. 4G).

SEM analysis corroborates the damage suggested by fluorescence microscopy. As revealed by fluorescence microscopy, *K. pneumoniae* cells present pores in the membrane after treatment with *Mo*-CBP$_3$-PepI. SEM analysis revealed several damages in the structure of *K. pneumoniae*, including loss of internal content mediated by pores. Mo-CBP3-PepI induced the same damage in *Candida* cells [16]. For example, *Mo*-CBP$_3$-PepIII, a closely related peptide of *Mo*-CBP$_3$-PepI, also damaged the morphology of *Staphylococcus aureus* [15].

3.3. Proteomic profile of K. pneumoniae cells treated with Mo-CBP$_3$-PepI

3.3.1. Overview

Proteomic analysis is a powerful technique employed to overview what happens in cells after treatment with peptides [35–39]. Proteomic response to peptides has been analyzed in *Escherichia coli* K12 [40], *Bacillus subtilis* [35], and *Clostridioides difficile* [38]. In many cases, proteomic analysis has been performed to understand the behavior of multidrug resistance bacteria response to antibiotics [36]. Here, proteomic analysis was employed to overview protein changes in *K. pneumoniae* after treatment with *Mo*-CBP$_3$-PepI. In total, 547 were successfully identified (Fig. 5). Of these, 279 proteins were identified in *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI and 268 from control *K. pneumoniae* cells (Fig. 3A and Supplementary Tables 1 and 2). Yet, *Mo*-CBP$_3$-PepI-treated *K. pneumoniae* cells presented 232 unique proteins only identified in this group and 221 unique from control *K. pneumoniae* cells (Fig. 5A and Table 1).

Besides the unique proteins, which are those exclusively found in one group, there were proteins detected in both groups, and they were shared proteins. To understand the patterns of differential accumulation of these proteins, a fold-change rule was applied, taking into account the intensity of protein *Mo*-CBP$_3$-PepI/Control *K pneumoniae* cells. Proteins with a fold-change value ≥ 1.5 ($p<0.05$, Tukey's test) [41] were considered up-accumulated (increased the abundance), and proteins with a fold-change value ≤ 0.5 ($p<0.05$, Tukey's test) were considered down-accumulated (decreased the abundance) were taken into consideration for comparisons. Forty-seven proteins common to both groups, 19 up-accumulated, 19 down-accumulated, and 9, did not change when comparing *Mo*-CBP$_3$-PepI-treated with control cells (Fig. 5B).

The Gene ontology classification of proteins shared by *Mo*-CBP3-PepI- and control-*K. pneumoniae* cells revealed 11 and 16 groups of proteins regarding, respectively, biological activity and molecular function (Fig. 6). Regarding the biological activity, the group that held the highest number of identified proteins was Energy and metabolism with 36% of total proteins and DNA metabolism group held the lowest number with 2% of total identified proteins (Fig. 5). In case of molecular function, the transferase group possesses the highest amount, 26%, of identified proteins. Many groups, such as chaperone and ion binding, have a small number of proteins, 2% of the total identified included (Fig. 6).

The protein groups involved in regulating transcription, transmembrane transporters, stress, and Defense Response, Energy and Metabolism, Pathogenesis, Protein Biosynthesis, Metabolism, Cell wall organization, and structural maintenance and transferase were composed of proteins that are both up- and down-accumulated (Table 2). In contrast, the groups Regulation Factor and RNA Processing and DNA metabolism are composed

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by proteins that decrease the abundance in *K. pneumoniae* cells after treatment with *Mo*-CBP$_3$-PepI (Table 1 and Fig. 5B). On the other hand, the down-regulated proteins were related to regulation factors and transferase (Fig. 5 - Heatmap).

3.3.2. DNA metabolism-related proteins

In this group, one protein was found in both *Mo*-CBP$_3$-PepI and DMSO groups, Endonuclease 8 (Table 2), which was down-accumulated in *K. pneumoniae* cells treated *Mo*-CBP$_3$-PepI compared to DMSO cells (control). Endonuclease 8 is an enzyme involved in the DNA repair process after damage by oxidation by ROS [42–44]. The reduction in the abundance of Endonuclease 8 in cells treated with *Mo*-CBP$_3$-PepI is interesting because it noticed a higher accumulation of ROS (Fig. 3) in those cells. So, the high accumulation of ROS and reduction in accumulation the Endonuclease 8 suggest that DNA from *K. pneumoniae* cells is being damaged by ROS induced by *Mo*-CBP$_3$-PepI [26].

By analyzing proteins exclusively (Table Supplementary 1) detected in *K. pneumoniae* cells after treatment with *Mo*-CBP$_3$-PepI and absent in control cells such as UvrABC system protein A, UvrABC system protein B, and UvrABC system protein C is clear to notice that *Mo*-CBP$_3$-PepI induce several and different types of damage in DNA of *K. pneumoniae* because these enzymes are involved damage in both strands of DNA [45]. Interestingly, only in control cells (Table Supplementary 2), but not in treated cells, the protein DNA repair protein RecN was detected as the first line of the cell to protect DNA from damage [46]. Somehow, *Mo*-CBP$_3$-PepI induces a down-accumulation in *K. pneumoniae* cells that, combined with ROS accumulation, leads to DNA damage and cell death.

3.3.3. Stress and Defense Response related proteins

In this group, one protein deserved attention, Peptide methionine sulfoxide reductase MsrB was highly accumulated in *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI compared to control cells with a fold-change value of 14.104 (Table 1). The MsrB protein is a highly conserved protein essential in the cell defense mechanism against high ROS levels, and it works in the repair of inactivated protein by ROS [47]. The increase in MsrB in cells treated with *Mo*-CBP$_3$-PepI compared to control agrees with the high accumulation of ROS in cells (Fig. 3). Proteins and other vital molecules in the cell are attacked and inactivated by ROS [26]. The fold-change value presented by MsrB protein indicates severe damage in proteins of *K. pneumoniae* cells after treatment *Mo*-CBP$_3$-PepI and that cells are trying to recover from this stress.

Proteomic analysis of cells revealed a reduction in the accumulation of an important protein, Lon protease, in cells treated with *Mo*-CBP$_3$-PepI compared to control cells (Table 2). Lon protease is a multifunctional, highly conserved ATP-dependent serine protease involved in protein turnover in bacterial cells [48,49]. Lon protease degrades either natural or ROS-induced misfolded proteins leading to free amino acids to produce new functional proteins [48,50]. The reduction in the abundance of Lon protease in *Mo*-CBP$_3$-PepI -treated cells may suggest an accumulation of misfolded proteins that mitigate the chance of responding to the stress imposed by *Mo-CBP3-PepI*. Additionally, Lon protease is essential to encapsulation, motility, heat-shock response, persister formation and drug resistance, and virulence factor production [51–56]. By inducing the reduction of accumulation of Lon protease in *K. pneumoniae* cells, *Mo-CBP3-PepI* dramatically reduced the chances of the cell to respond to stress and inhibit several essential processes to cell normal function leading to death.

Looking at proteins unique from control (Supplementary Table 3) cells, proteins such as multidrug resistance protein MdtN, UPF0194 membrane protein YbhG, and multidrug resistance protein EmrK were exclusive of control cells being absent in *Mo*-CBP$_3$-PepI. MdtN is a protein involved in the resistance against puromycin and acriflavine [55], EmrK is a part of the efflux pump involved in multidrug resistance [57], and YbhG is involved in resistance to chloramphenicol [58]. This is an exciting result because the absence of these in *Mo-CBP3-PepI* treated cells indicates that they became susceptible to these

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antibiotics again, which is an important outcome. 3.3.4. Protein Biosynthesis and Metabolism related proteins

The analysis of proteins related to protein biosynthesis and metabolism revealed a quite complex scenario in *K. pneumoniae* cells after treatment with *Mo*-CBP$_3$-PepI (Table 2 and Supplmentary tables 1 and 2). For example, among the overlapping proteins, the 50S ribosomal protein L22 and 50S ribosomal protein L7/L12, respectively, showed a reduction and increased abundance in *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI compared to control cells (Table 2). The 50S ribosomal protein L22 is a vital core protein of bacterial ribosomes involved in the aggregation and stabilization of ribosomal proteins to form the ribosome in bacteria [59,60]. The L22 subunit is so important to bacterial ribosomes that it is the target of antibiotics such as macrolides [59,60]. The reduction in abundance in this protein induced by *Mo-CBP3-PepI* indicates a destabilization of bacterial ribosomes leading to the inhibition of protein synthesis in bacteria.

It was seen an increase in the abundance of 50S ribosomal protein L7/L12 in cells treated with *Mo-CBP3-PepI* (Table 2) compared to the control. The increase of this protein is maybe a mechanism of the cell to supply the deficiency of the ribosomal protein L22. However, the L7/L12 is a GTPase protein involved in the process such as translation initiation, elongation, and termination by the mature 70S ribosome [61]. However, the increase in this protein will not help cells perform protein synthesis without the L22 subunit.

An interesting result came out by evaluating the unique proteins, either *Mo*-CBP$_3$-PepI and control cells (Supplmentary tables 1 and 2). Among the proteins exclusively detected in *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI are Cysteine-tRNA ligase, Leucine-tRNA ligase, Serine-tRNA ligase, Valine-tRNA ligase, Glutamine-tRNA, Phenylalanine--tRNA ligase alpha subunit, Valine--tRNA ligase, Proline--tRNA ligase, Alanine--tRNA ligase, and Threonine--tRNA ligase (Supplementary table 1). All these proteins are involved in amino acid delivery to ribosomes during protein synthesis. The increase in these proteins' abundance indicates a cell attempt to either increase or maintain the protein synthesis in normal levels to allow cells to fight back against insults imposed by *Mo-CBP3-PepI*.

Proteins are important to all living organisms, and bacteria must understand what is happening in their environment to respond accordingly. Proteins make all this. To respond to stress agents, such as *Mo*-CBP3-PepI, *K. pneumoniae* must reprogram all its protein profiles to produce defense proteins [62]. For example, *K. pneumoniae* cells should produce scavenger proteins to defend themselves from ROS overproduction, but that is impossible. That happens because *Mo-CBP3-PepI* reduced the abundance of an important protein for ribosomal activity. As a consequence, *K. pneumoniae* cells could not produce scavenger proteins leading to ROS accumulation and damage to DNA (as reported above) and damage to other proteins leading the cell to death as revealed by damage present in fluorescence and scanning electron microscopy.

3.3.5. Regulation Factor and RNA Processing related proteins

In this group of proteins, the protein elongation factor (Q7NAV3) G that decreases in abundance in *K. pneumoniae* treated with *Mo-CBP3-PepI* deserves attention (Table 2). By looking into unique proteins from control cells (supplementary table 3), one isoform of elongation factor G (Q492B1) and other factors, such as Elongation factor Tu 1 and Elongation factor 4, is present but disappear completely in treat cells. Elongation factor G is important for the translocation process during prokaryotic protein synthesis, and it uses the Energy held in GTP to interact with tRNA and mRNA [63]. The decrease in the abundance of Elongation factor G led to the shutdown of protein synthesis in *K. pneumoniae* cells. As discussed above in the protein metabolism section, the protein synthesis in *K. pneumoniae* cells is dramatically affected by Mo-CBP3-PepI, giving them no chance to respond to the stress imposed by the peptide.

3.3.6. Cell wall organization and structure maintenance related proteins

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In this group of proteins, the most important were exclusively found in *K. pneumoniae* cells treated with *Mo*-CBP3-PepI (Supplementary Table 1). Among those, Soluble lytic murein transglycosylase, D-alanyl-D-alanine carboxypeptidase DacB, Cell shape-determining protein MreB, Probable L, D-transpeptidase ErfK/SrfK, Cell shape-determining protein MreC, Murein tetrapeptide carboxypeptidase, Murein DD-endopeptidase MepH, Sensor protein LytS, D-alanine--D-alanine ligase, and Inner membrane protein YdcZ. All these proteins are involved in cell wall turnover, cell structure maintenance, and shape stabilization [64–66]. The increased abundance of these proteins indicates that K. pneumoniae cells are suffering from stress in the cell wall imposed by *Mo*-CBP3-PepI and are trying to overcome the stress. However, as revealed by SEM analysis (Fig. 4), *K. pneumoniae* cells treated presented several damages to the cell wall and altered cell morphology. It has been related that *Mo*-CBP3-PepI can bind to the cell wall of yeast *C. albicans* [16].

The bacterial cell wall is a crucial component of the cell involved in mechanical defense upon several environmental stresses [67]. During stress, the cell must recover the cell wall every time damage occurs. The exclusive detection of this protein in cells treated with *Mo*-CBP$_3$-PepI indicates that it is imposing stress on the cell wall and is trying to recover, but as revealed by microscopic analysis (Figs. 3 and 4).

3.3.7. Transferase-related proteins

In this group, one protein is relevant, the sensor histidine kinase HprS, which presented a reduced abundance in cells treated with *Mo-CBP3-PepI* compared to control cells (Table 2). Histidine kinase sensors are essential elements bacteria use to sense the environment and respond accordingly [68,69]. These sensors are mainly responsible for perceiving and responding to oxidative stress [70]. Here, the reduction in the abundance of HprS agreed with the high accumulation of ROS (Fig. 3) in *K. pneumoniae* cells treated with *Mo-CBP3-PepI*. This result shows that *Mo-CBP3-PepI* imposes two stresses on *K. pneumoniae* cells. First is the induction of accumulation of ROS at higher levels; second is the reduction in the protein accumulation involved in the perception and response of stress caused by ROS. In this case, *Mo*-CBP3-PepI simultaneously induces stress and inhibits the cell's ability to develop a response to it.

3.3.8. Cell Redox Homeostasis related proteins

This group is particularly important given the scenario of high levels of ROS accumulation in *K. pneumoniae* cells induced by *Mo-CBP3-PepI*. In this group, were no overlapping proteins found, only exclusive proteins either from *Mo-CBP3-PepI*-treated or control *K. pneumoniae* cells (Supplementary Tables 1 and 2). For example, Alkyl hydroperoxide reductase C and Alkyl hydroperoxide reductase subunit F were only detected in control *K. pneumoniae* (Supplementary Tables 2). These enzymes are thiol-peroxidases and the first line of cell defense responsible for the scavenging of H_2O_2 displaying a critical role in protecting the bacterial cell from endogenously H_2O_2 [71,72].

Interestingly, these enzymes were not detected in cells treated with *Mo*-CBP$_3$-PepI, indicating a complete depletion of these enzymes. The absence of these enzymes in treated cells might lead to the accumulation of H_2O_2 (Fig. 3), as revealed by fluorescence microscopy. To cope with the high levels of ROS induced by peptides, *K. pneumoniae* cells increase the abundance of a catalase-peroxidase enzyme, only detected in treated cells, which is involved in the defense mechanism against high levels of ROS [71,73]. Our data revealed that even though *K. pneumoniae* cells treated with *Mo*-CBP$_3$-PepI increased the abundance of a catalase enzyme to remove the excess ROS. However, it's not enough to prevent the damage produced by them once the microscopies show damage to cell structure and many enzymes suggest damage in DNA and proteins.

The mechanisms by which *Mo*-CBP$_3$-PepI induced damage and death in *K. pneumoniae* cells are many and quite complex. Based on that, a scheme (Fig. X) was produced to provide an overview of all processes induced by *Mo*-CBP$_3$-PepI that lead K. pneumoniae to death. (1) As revealed by PI uptake, *Mo*-CBP$_3$-PepI can induce the formation of

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small pores on the membrane of *K. pneumoniae*. Additionally, FITC-Dextran revealed the presence 10-kDa sized pore (2). (3) the interaction of *Mo*-CBP$_3$-PepI lead to the accumulation of high levels of ROS inside the cell. (4) ROS accumulation led to damage to DNA. (5) The high levels of ROS led to damage to proteins leading to misfolding and degradation. (6) as revealed by proteomic analysis, *Mo*-CBP$_3$-PepI induced a reduction in the abundance of proteins related to proteins and, together with events in (5), led to a shutdown in protein in *K. pneumoniae* cells. (7) The shutdown in protein inhibits the cell wall turnover leading to damage, as revealed by SEM analysis (Fig. 4). (8) the shutdown in protein levels is also responsible for reducing proteins related to antibiotic resistance. (9) there is also a reduction in a group of proteins involved in early response to ROS accumulation leading to the accumulation of ROS at higher levels. (10) The higher levels of ROS led to damage to DNA (3), proteins (5), and lipids on the membrane (10).

4. Conclusion

Altogether the data presented here indicate an intricated and coordinated sequence of events induced by *Mo*-CBP$_3$-PepI that cloud drive *K. pneumoniae* cells to death. All these complex mechanisms of action are also difficult for *K. pneumoniae* to develop resistance because they present multiple targets simultaneously. Based on that, it is feasible to suggest that our peptide is a potential candidate for developing new strategies to cope with *K. pneumoniae* resistance. L.A.C.B, P.F.N.S., N.A.S.N., T.K.B.A. A.F.B.S. R.F. C. C.S.N. F.P.M. L.B.L. C.D.T.F.

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References

1. O'Neill, J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations 2016.
2. About Antibiotic Resistance | CDC Available online: https://www.cdc.gov/drugresistance/about.html (accessed on 11 November 2022).
3. Murray, C.J.; Ikuta, K.S.; Sharara, F.; Swetschinski, L.; Robles Aguilar, G.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; et al. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *Lancet* **2022**, *399*, 629–655, doi:10.1016/S0140-6736(21)02724-0.
4. Klebsiella Pneumoniae in Healthcare Settings | HAI | CDC Available online: https://www.cdc.gov/hai/organisms/klebsiella/klebsiella.html (accessed on 11 November 2022).
5. Mulani, M.S.; Kamble, E.E.; Kumkar, S.N.; Tawre, M.S.; Pardesi, K.R. Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review. *Front. Microbiol.* **2019**, *10*, 539, doi:10.3389/FMICB.2019.00539.
6. De Oliveira, D.M.P.; Forde, B.M.; Kidd, T.J.; Harris, P.N.A.; Schembri, M.A.; Beatson, S.A.; Paterson, D.L.; Walker, M.J. Antimicrobial Resistance in ESKAPE Pathogens. *Clin. Microbiol. Rev.* **2020**, *33*, doi:10.1128/CMR.00181-19/ASSET/CBA1C1D1-CF90-43DE-A9DF-32D24A4334AC/ASSETS/GRAPHIC/CMR.00181-19-F0001.JPEG.
7. Livermore, D.M.; Macgowan, A.P.; Wale, M.C.J. *Surveillance of Antimicrobial Resistance*; 1998; Vol. 317; ISBN 9789294983879.
8. Tzouvelekis, L.S.; Markogiannakis, A.; Piperaki, E.; Souli, M.; Daikos, G.L. Treating Infections Caused by Carbapenemase-Producing Enterobacteriaceae. *Clin. Microbiol. Infect.* **2014**, *20*, 862–872, doi:10.1111/1469-0691.12697.
9. Zowawi, H.M.; Forde, B.M.; Alfaresi, M.; Alzarouni, A.; Farahat, Y.; Chong, T.M.; Yin, W.F.; Chan, K.G.; Li, J.; Schembri, M.A.; et al. Stepwise Evolution of Pandrug-Resistance in Klebsiella Pneumoniae. *Sci. Rep.* **2015**, *5*, 1–8, doi:10.1038/srep15082.
10. LA, O.; A, S.; JS, B. 乳鼠心肌提取 HHS Public Access. *Physiol. Behav.* **2017**, *176*, 139–148, doi:10.1097/INF.0000000000001991.Osteoarticular.

11. Corey, G.R.; Arhin, F.F.; Wikler, M.A.; Sahm, D.F.; Kreiswirth, B.N.; Mediavilla, J.R.; Good, S.; Fiset, C.; Jiang, H.; Moeck, G.; et al. Pooled Analysis of Single-Dose Oritavancin in the Treatment of Acute Bacterial Skin and Skin-Structure Infections Caused by Gram-Positive Pathogens, Including a Large Patient Subset with Methicillin-Resistant Staphylococcus Aureus. *Int. J. Antimicrob. Agents* **2016**, *48*, 528–534, doi:10.1016/j.ijantimicag.2016.07.019.
12. Rubinstein, E.; Kollef, M.H.; Nathwani, D. Pneumonia Caused by Methicillin-Resistant Staphylococcus Aureus. *Clin. Infect. Dis.* **2008**, *46*, doi:10.1086/533594.
13. Lima, A.M.; Azevedo, M.I.G.; Sousa, L.M.; Oliveira, N.S.; Andrade, C.R.; Freitas, C.D.T.; Souza, P.F.N. Plant Antimicrobial Peptides: An Overview about Classification, Toxicity and Clinical Applications. *Int. J. Biol. Macromol.* **2022**, *214*, 10–21, doi:10.1016/j.ijbiomac.2022.06.043.
14. Souza, P.F.N.; Marques, L.S.M.; Oliveira, J.T.A.; Lima, P.G.; Dias, L.P.; Neto, N.A.S.; Lopes, F.E.S.; Sousa, J.S.; Silva, A.F.B.; Caneiro, R.F.; et al. Synthetic Antimicrobial Peptides: From Choice of the Best Sequences to Action Mechanisms. *Biochimie* **2020**, *175*, 132–145, doi:10.1016/j.biochi.2020.05.016.
15. Oliveira, J.T.A.; Souza, P.F.N.; Vasconcelos, I.M.; Dias, L.P.; Martins, T.F.; Van Tilburg, M.F.; Guedes, M.I.F.; Sousa, D.O.B. Mo-CBP3-PepI, Mo-CBP3-PepII, and Mo-CBP3-PepIII Are Synthetic Antimicrobial Peptides Active against Human Pathogens by Stimulating ROS Generation and Increasing Plasma Membrane Permeability. *Biochimie* **2019**, *157*, 10–21, doi:10.1016/j.biochi.2018.10.016.
16. Lima, P.G.; Souza, P.F.N.; Freitas, C.D.T.; Oliveira, J.T.A.; Dias, L.P.; Neto, J.X.S.; Vasconcelos, I.M.; Lopes, J.L.S.; Sousa, D.O.B. Anticandidal Activity of Synthetic Peptides: Mechanism of Action Revealed by Scanning Electron and Fluorescence Microscopies and Synergism Effect with Nystatin. *J. Pept. Sci.* **2020**, 1–13, doi:10.1002/psc.3249.
17. Souza, P.F.N.; Lima, P.G.; Freitas, C.D.T.; Sousa, D.O.B.; Neto, N.A.S.; Dias, L.P.; Vasconcelos, I.M.; Freitas, L.B.N.; Silva, R.G.G.; Sousa, J.S.; et al. Antidermatophytic Activity of Synthetic Peptides: Action Mechanisms and Clinical Application as Adjuvants to Enhance the Activity and Decrease the Toxicity of Griseofulvin. *Mycoses* **2020**, *63*, 979–992, doi:10.1111/myc.13138.
18. Neto, N.A.S.; Oliveira, J.T.A.; Aguiar, T.K.B.; Bezerra, L.P.; Branco, L.A.C.; Mesquita, F.P.; Freitas, C.D.T.; Souza, P.F.N. Synergistic Antibiofilm Activity between Synthetic Peptides and Ciprofloxacin against Staphylococcus Aureus. *Pathogens* **2022**, *11*, 995, doi:10.3390/pathogens11090995.
19. Lima, P.G.; Freitas, C.D.T.; Oliveira, J.T.A.; Neto, N.A.S.; Amaral, J.L.; Silva, A.F.B.; Sousa, J.S.; Franco, O.L.; Souza, P.F.N. Synthetic Antimicrobial Peptides Control Penicillium Digitatum Infection in Orange Fruits. *Food Res. Int.* **2021**, *147*, doi:10.1016/j.foodres.2021.110582.
20. Staniszewska, M.; Bondaryk, M.; Swoboda-Kopec, E.; Siennicka, K.; Sygitowicz, G.; Kurzatkowski, W. Candida Albicans Morphologies Revealed by Scanning Electron Microscopy Analysis. *Brazilian J. Microbiol.* **2013**, *44*, 813–821, doi:10.1590/S1517-83822013005000056.
21. Fleeman, R.M.; Macias, L.A.; Brodbelt, J.S.; Davies, B.W. Defining Principles That Influence Antimicrobial Peptide Activity against Capsulated Klebsiella Pneumoniae. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 27620–27626, doi:10.1073/PNAS.2007036117/-/DCSUPPLEMENTAL.
22. Tincho, M.B.; Morris, T.; Meyer, M.; Pretorius, A. Antibacterial Activity of Rationally Designed Antimicrobial Peptides. *Int. J. Microbiol.* **2020**, *2020*, doi:10.1155/2020/2131535.
23. Jindal, H.M.; Le, C.F.; Yusof, M.Y.M.; Velayuthan, R.D.; Lee, V.S.; Zain, S.M.; Isa, D.M.; Sekaran, S.D. Antimicrobial Activity of Novel Synthetic Peptides Derived from Indolicidin and Ranalexin against Streptococcus Pneumoniae. *PLoS One* **2015**, *10*, doi:10.1371/JOURNAL.PONE.0128532.
24. Parra, A.L.C.; Freitas, C.D.T.; Souza, P.F.N.; von Aderkas, P.; Borchers, C.H.; Beattie, G.A.; Silva, F.D.A.; Thornburg, R.W. Ornamental Tobacco Floral Nectar Is a Rich Source of Antimicrobial Peptides. *Plant Sci.* **2022**, *324*, 111427, doi:10.1016/j.plantsci.2022.111427.
25. Aguiar, T.K.B.; Neto, N.A.S.; Freitas, C.D.T.; Silva, A.F.B.; Bezerra, L.P.; Malveira, E.A.; Branco, L.A.C.; Mesquita, F.P.; Goldman, G.H.; Alencar, L.M.R.; et al. Antifungal Potential of Synthetic Peptides against Cryptococcus Neoformans: Mechanism of Action Studies Reveal Synthetic Peptides Induce Membrane–Pore Formation, DNA Degradation, and Apoptosis. *Pharmaceutics* **2022**, *14*, 1678, doi:10.3390/pharmaceutics14081678.
26. Wang, K.; Dang, W.; Xie, J.; Zhu, R.; Sun, M.; Jia, F.; Zhao, Y.; An, X.; Qiu, S.; Li, X.; et al. Antimicrobial Peptide Protonectin Disturbs the Membrane Integrity and Induces ROS Production in Yeast Cells. *Biochim. Biophys. Acta - Biomembr.* **2015**, *1848*, 2365–2373, doi:10.1016/J.BBAMEM.2015.07.008.
27. Lee, M.T.; Hung, W.C.; Chen, F.Y.; Huang, H.W. Mechanism and Kinetics of Pore Formation in Membranes by Water-Soluble Amphipathic Peptides. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 5087–5092, doi:10.1073/PNAS.0710625105/SUPPL_FILE/0710625105MOVIE01.MOV.
28. Lima, P.G.; Oliveira, J.T.A.; Amaral, J.L.; Freitas, C.D.T.; Souza, P.F.N. Synthetic Antimicrobial Peptides: Characteristics, Design, and Potential as Alternative Molecules to Overcome Microbial Resistance. *Life Sci.* **2021**, *278*, 119647, doi:10.1016/J.LFS.2021.119647.
29. Hristova, K.; Wimley, W.C. A Look at Arginine in Membranes. *J. Membr. Biol.* **2011**, *239*, 49, doi:10.1007/S00232-010-9323-9.
30. Campelo, F.; McMahon, H.T.; Kozlov, M.M. The Hydrophobic Insertion Mechanism of Membrane Curvature Generation by Proteins. *Biophys. J.* **2008**, *95*, 2325–2339, doi:10.1529/BIOPHYSJ.108.133173.
31. Huang, Y.; Huang, J.; Chen, Y. Alpha-Helical Cationic Antimicrobial Peptides: Relationships of Structure and Function. *Protein Cell* **2010**, *1*, 143–152, doi:10.1007/s13238-010-0004-3.
32. Shai, Y. Mechanism of the Binding, Insertion and Destabilization of Phospholipid Bilayer Membranes by Alpha-Helical Antimicrobial and Cell Non-Selective Membrane-Lytic Peptides. *Biochim. Biophys. Acta* **1999**, *1462*, 55–70.

33. Jean-François, F.; Elezgaray, J.; Berson, P.; Vacher, P.; Dufourc, E.J. Pore Formation Induced by an Antimicrobial Peptide: Electrostatic Effects. *Biophys. J.* **2008**, *95*, 5748–5756, doi:10.1529/biophysj.108.136655.
34. Rowe-Magnus, D.A.; Kao, A.Y.; Prieto, A.C.; Pu, M.; Kao, C. Cathelicidin Peptides Restrict Bacterial Growth via Membrane Perturbation and Induction of Reactive Oxygen Species. *MBio* **2019**, *10*, 1–19, doi:10.1128/mBio.02021-19.
35. Wenzel, M.; Chiriac, A.I.; Otto, A.; Zweytick, D.; May, C.; Schumacher, C.; Gust, R.; Albada, H.B.; Penkova, M.; Krämer, U.; et al. Small Cationic Antimicrobial Peptides Delocalize Peripheral Membrane Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 1409–1418, doi:10.1073/pnas.1319900111.
36. Tsakou, F.; Jersie-Christensen, R.; Jenssen, H.; Mojsoska, B. The Role of Proteomics in Bacterial Response to Antibiotics. *Pharmaceuticals* **2020**, *13*, 1–27, doi:10.3390/ph13090214.
37. Moyer, T.B.; Purvis, A.L.; Wommack, A.J.; Hicks, L.M. Proteomic Response of Escherichia Coli to a Membrane Lytic and Iron Chelating Truncated Amaranthus Tricolor Defensin. *BMC Microbiol.* **2021**, *21*, 1–12, doi:10.1186/s12866-021-02176-4.
38. Maaß, S.; Bartel, J.; Mücke, P.A.; Schlüter, R.; Sura, T.; Zaschke-Kriesche, J.; Smits, S.H.J.; Becher, D. Proteomic Adaptation of Clostridioides Difficile to Treatment with the Antimicrobial Peptide Nisin. *Cells* **2021**, *10*, 1–21, doi:10.3390/cells10020372.
39. Senges, C.H.R.; Stepanek, J.J.; Wenzel, M.; Raatschen, N.; Ay, Ü.; Märtens, Y.; Prochnow, P.; Hernández, M.V.; Yayci, A.; Schubert, B.; et al. Comparison of Proteomic Responses as Global Approach to Antibiotic Mechanism of Action Elucidation. *Antimicrob. Agents Chemother.* **2021**, *65*, doi:10.1128/AAC.01373-20.
40. Wang, H.; Xie, Y.; Zhang, H.; Jin, J.; Zhang, H. Quantitative Proteomic Analysis Reveals the Influence of Plantaricin BM-1 on Metabolic Pathways and Peptidoglycan Synthesis in Escherichia Coli K12. *PLoS One* **2020**, *15*, 1–13, doi:10.1371/journal.pone.0231975.
41. Noronha Souza, P.F.; Abreu Oliveira, J.T.; Vasconcelos, I.M.; Magalhães, V.G.; Albuquerque Silva, F.D.; Guedes Silva, R.G.; Oliveira, K.S.; Franco, O.L.; Gomes Silveira, J.A.; Leite Carvalho, F.E. H2O2Accumulation, Host Cell Death and Differential Levels of Proteins Related to Photosynthesis, Redox Homeostasis, and Required for Viral Replication Explain the Resistance of EMS-Mutagenized Cowpea to Cowpea Severe Mosaic Virus. *J. Plant Physiol.* **2020**, *245*, 153110, doi:10.1016/j.jplph.2019.153110.
42. Burgess, S.; Jaruga, P.; Dodson, M.L.; Dizdaroglu, M.; Stephen Lloyd, R. Determination of Active Site Residues in Escherichia Coli Endonuclease VIII. *J. Biol. Chem.* **2002**, *277*, 2938–2944, doi:10.1074/JBC.M110499200.
43. Jiang, D.; Hatahet, Z.; Blaisdell, J.O.; Melamede, R.J.; Wallace, S.S. Escherichia Coli Endonuclease VIII: Cloning, Sequencing, and Overexpression of the Nei Structural Gene and Characterization of Nei and Nei Nth Mutants. *J. Bacteriol.* **1997**, *179*, 3773–3782, doi:10.1128/JB.179.11.3773-3782.1997.
44. Oshima, T.; Aiba, H.; Baba, T.; Fujita, K.; Hayashi, K.; Honjo, A.; Ikemoto, K.; Inada, T.; Itoh, T.; Kajihara, M.; et al. A 718-Kb DNA Sequence of the Escherichia Coli K-12 Genome Corresponding to the 12.7–28.0 Min Region on the Linkage Map. *DNA Res.* **1996**, *3*, 137–155, doi:10.1093/DNARES/3.3.137.
45. Verhoeven, E.E.A.; Wyman, C.; Moolenaar, G.F.; Goosen, N. The Presence of Two UvrB Subunits in the UvrAB Complex Ensures Damage Detection in Both DNA Strands. *EMBO J.* **2002**, *21*, 4196–4205, doi:10.1093/EMBOJ/CDF396.
46. Kidane, D.; Sanchez, H.; Alonso, J.C.; Graumann, P.L. Visualization of DNA Double-Strand Break Repair in Live Bacteria Reveals Dynamic Recruitment of Bacillus Subtilis RecF, RecO and RecN Proteins to Distinct Sites on the Nucleoids. *Mol. Microbiol.* **2004**, *52*, 1627–1639, doi:10.1111/J.1365-2958.2004.04102.X.
47. Delaye, L.; Becerra, A.; Orgel, L.; Lazcano, A. Molecular Evolution of Peptide Methionine Sulfoxide Reductases (MsrA and MsrB): On the Early Development of a Mechanism That Protects against Oxidative Damage. *J. Mol. Evol.* **2007**, *64*, 15–32, doi:10.1007/S00239-005-0281-2.
48. Luo, S.; McNeill, M.; Myers, T.G.; Hohman, R.J.; Levine, R.L. Lon Protease Promotes Survival of Escherichia Coli During Anaerobic Glucose Starvation. *Arch. Microbiol.* **2008**, *189*, 181, doi:10.1007/S00203-007-0304-Z.
49. He, L.; Luo, D.; Yang, F.; Li, C.; Zhang, X.; Deng, H.; Zhang, J.R. Multiple Domains of Bacterial and Human Lon Proteases Define Substrate Selectivity. *Emerg. Microbes Infect.* **2018**, *7*, doi:10.1038/S41426-018-0148-4.
50. Stadtman, E.R.; Levine, R.L. Free Radical-Mediated Oxidation of Free Amino Acids and Amino Acid Residues in Proteins. *Amino Acids* **2003**, *25*, 207–218, doi:10.1007/S00726-003-0011-2.
51. Torres-Cabassa, A.; Gottesman, S.; Frederick, R.D.; Dolph, P.J.; Coplin, D.L. Control of Extracellular Polysaccharide Synthesis in Erwinia Stewartii and Escherichia Coli K-12: A Common Regulatory Function. *J. Bacteriol.* **1987**, *169*, 4525, doi:10.1128/JB.169.10.4525-4531.1987.
52. Mukherjee, S.; Bree, A.C.; Liu, J.; Patrick, J.E.; Chien, P.; Kearns, D.B. Adaptor-Mediated Lon Proteolysis Restricts Bacillus Subtilis Hyperflagellation. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 250–255, doi:10.1073/PNAS.1417419112/-/DCSUPPLEMENTAL.
53. Bissonnette, S.A.; Rivera-Rivera, I.; Sauer, R.T.; Baker, T.A. The IbpA and IbpB Small Heat-Shock Proteins Are Substrates of the AAA+ Lon Protease. *Mol. Microbiol.* **2010**, *75*, 1539, doi:10.1111/J.1365-2958.2010.07070.X.
54. Shan, Y.; Gandt, A.B.; Rowe, S.E.; Deisinger, J.P.; Conlon, B.P.; Lewis, K. ATP-Dependent Persister Formation in Escherichia Coli. *MBio* **2017**, *8*, doi:10.1128/MBIO.02267-16.
55. Ricci, V.; Blair, J.M.A.; Piddock, L.J.V. RamA, Which Controls Expression of the MDR Efflux Pump AcrAB-TolC, Is Regulated by the Lon Protease. *J. Antimicrob. Chemother.* **2014**, *69*, 643, doi:10.1093/JAC/DKT432.
56. Herbst, K.; Bujara, M.; Heroven, A.K.; Opitz, W.; Weichert, M.; Zimmermann, A.; Dersch, P. Intrinsic Thermal Sensing Controls Proteolysis of Yersinia Virulence Regulator RovA. *PLoS Pathog.* **2009**, *5*, 1000435, doi:10.1371/JOURNAL.PPAT.1000435.
57. EmrK - Probable Multidrug Resistance Protein EmrK - Escherichia Coli (Strain K12) | UniProtKB | UniProt Available online: https://www.uniprot.org/uniprotkb/P52599/entry (accessed on 7 November 2022).
58. YbhG - UPF0194 Membrane Protein YbhG - Escherichia Coli (Strain K12) | UniProtKB | UniProt Available online: https://www.uniprot.org/uniprotkb/P75777/entry (accessed on 7 November 2022).

59. Moore, S.D.; Sauer, R.T. Revisiting the Mechanism of Macrolide-Antibiotic Resistance Mediated by Ribosomal Protein L22. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 18261–18266, doi:10.1073/pnas.0810357105.
60. Davydova, N.; Streltsov, V.; Wilce, M.; Liljas, A.; Garber, M. L22 Ribosomal Protein and Effect of Its Mutation on Ribosome Resistance to Erythromycin. *J. Mol. Biol.* **2002**, *322*, 635–644, doi:10.1016/S0022-2836(02)00772-6.
61. Carlson, M.A.; Haddad, B.G.; Weis, A.J.; Blackwood, C.S.; Shelton, C.D.; Wuerth, M.E.; Walter, J.D.; Spiegel, P.C. Ribosomal Protein L7/L12 Is Required for GTPase Translation Factors EF-G, RF3 and IF2 to Bind in Their GTP State to 70S Ribosomes. *FEBS J.* **2017**, *284*, 1631, doi:10.1111/FEBS.14067.
62. Starosta, A.L.; Lassak, J.; Jung, K.; Wilson, D.N. The Bacterial Translation Stress Response. *FEMS Microbiol. Rev.* **2014**, *38*, 1172, doi:10.1111/1574-6976.12083.
63. Chen, C.; Cui, X.; Beausang, J.F.; Zhang, H.; Farrell, I.; Cooperman, B.S.; Goldman, Y.E. Elongation Factor G Initiates Translocation through a Power Stroke. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 7515–7520, doi:10.1073/PNAS.1602668113/SUPPL_FILE/PNAS.201602668SI.PDF.
64. Divakaruni, A. V.; Baida, C.; White, C.L.; Gober, J.W. The Cell Shape Proteins MreB and MreC Control Cell Morphogenesis by Positioning Cell Wall Synthetic Complexes. *Mol. Microbiol.* **2007**, *66*, 174–188, doi:10.1111/j.1365-2958.2007.05910.x.
65. Dik, D.A.; Marous, D.R.; Fisher, J.F.; Mobashery, S. Lytic Transglycosylases: Concinnity in Concision of the Bacterial Cell Wall. *Crit. Rev. Biochem. Mol. Biol.* **2017**, *52*, 503–542, doi:10.1080/10409238.2017.1337705.
66. Hung, W.C.; Jane, W.N.; Wong, H.C. Association of a D-Alanyl-D-Alanine Carboxypeptidase Gene with the Formation of Aberrantly Shaped Cells during the Induction of Viable but Nonculturable Vibrio Parahaemolyticus. *Appl. Environ. Microbiol.* **2013**, *79*, 7305–7312, doi:10.1128/AEM.01723-13.
67. Mueller, E.A.; Levin, P.A. Bacterial Cell Wall Quality Control during Environmental Stress. *MBio* **2020**, *11*, 1–15, doi:10.1128/mBio.02456-20.
68. Szurmant, H.; Bu, L.; Brooks, C.L.; Hoch, J.A. An Essential Sensor Histidine Kinase Controlled by Transmembrane Helix Interactions with Its Auxiliary Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 5891–5896, doi:10.1073/pnas.0800247105.
69. Alkan, C.; Coe, P.; Eichler, E. 基因的改变 NIH Public Access. *Bone* **2011**, *23*, 1–7, doi:10.1002/prot.22485.Crystal.
70. Alkan, C.; Coe, P.; Eichler, E. 基因的改变 NIH Public Access. *Bone* **2011**, *23*, 1–7, doi:10.1111/mmi.12809.The.
71. Wang, H.W.; Chung, C.H.; Ma, T.Y.; Wong, H.C. Roles of Alkyl Hydroperoxide Reductase Subunit C (AhpC) in Viable but Nonculturable Vibrio Parahaemolyticus. *Appl. Environ. Microbiol.* **2013**, *79*, 3734–3743, doi:10.1128/AEM.00560-13.
72. Chen, L.; Xie, Q.W.; Nathan, C. Alkyl Hydroperoxide Reductase Subunit C (AhpC) Protects Bacterial and Human Cells against Reactive Nitrogen Intermediates. *Mol. Cell* **1998**, *1*, 795–805, doi:10.1016/S1097-2765(00)80079-9.
73. Yuan, F.; Yin, S.; Xu, Y.; Xiang, L.; Wang, H.; Li, Z.; Fan, K.; Pan, G. The Richness and Diversity of Catalases in Bacteria. *Front. Microbiol.* **2021**, *12*, 485, doi:10.3389/FMICB.2021.645477/BIBTEX.