Characterization of an OrtT-like toxin of *Salmonella enterica* serovar Houten

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Abstract

The *Escherichia coli* GhoT/GhoS system is a type V toxin–antitoxin system in which the antitoxin GhoS cleaves the GhoT mRNA, controlling its translation. GhoT is a small hydrophobic protein that damages bacterial membranes. OrtT is a GhoT-like toxin, but it apparently lacks a corresponding antitoxin and serves a different physiologic role. Using a profile hidden Markov model approach, a *Salmonella enterica* serovar Houten genome was screened to obtain homologs of GhoT/OrtT. We only found one protein (referred to here as OrtT-Sal) that shared more sequence identity with OrtT than GhoT. The chromosomal region around the coding sequence of OrtT-Sal suggests that it is an orphan toxin and can be under RpoH activation. To study OrtT-Sal, we chemically synthesized and expressed in *E. coli* the whole toxin and its N- and C-terminal regions (OrtT-Sal_{1–29} and OrtT-Sal_{29–57}, respectively). Our findings have shown that the overproduction of the polypeptides resulted in severe growth inhibition and cell lysis. Using circular dichroism, we found that OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} form an alpha-helical structure in the presence of SDS micelles or TFE. Finally, using carboxyfluorescein-loaded lipid vesicles, we determined that the polypeptides damage lipid membrane directly.

Keywords Toxin–antitoxin system · OrtT · GhoT · Orphan toxin

Introduction

Toxin–antitoxin (TA) systems are widespread in bacterial genomes, and they are found on plasmids and chromosomes. These genetic modules are commonly two-component systems that are composed of a toxin and an antitoxin [1, 2]. In such systems, the toxin causes repression of metabolism and growth arrest by interfering in an essential cellular process.

Antitoxin is responsible for neutralizing toxin activity during normal growth conditions. Under unfavorable conditions, especially under conditions of stress, antitoxins are depleted, allowing toxins to become free and to exert their toxic effect [1, 2]. In this context, toxins from different TA systems may have variable cell targets, including DNA replication machinery [3–5], mRNA [6, 7], cytoskeleton proteins [8], and membranes [9–12].

TA systems were initially discovered as a mechanism for plasmid stabilization during cell division [13, 14]. Later, TA systems were also found on the chromosomes of bacteria and archaea [15]. Studies have shown that these TA systems of chromosomal loci may play important roles in the cellular physiology of prokaryotes, such as persistent cell formation (cells with tolerance to antibiotics) [16], biofilm formation [17, 18], phage inhibition [19], and general stress response [20–22]. Knowledge about the mechanisms of action of TA systems has been used in the development of biotechnological products in molecular biology [23, 24]. Besides that, the importance of TA systems in persistence makes them potential targets for the development of new antibacterial drug [2, 25–27].

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Currently, TA systems are grouped into seven types [12] based on how the antitoxin neutralizes the toxin. In type I, the antitoxin is a noncoding RNA that acts on toxin mRNAs via a base-pairing mechanism to prevent their synthesis [28–31]. In type II, both the toxin and antitoxin are translated to produce proteins and the antitoxin tightly binds and inhibits the activity of the toxin [15, 32]. In type III, antitoxin is a small RNA that forms pseudoknots and that binds directly to the protein toxin [33, 34]. In type IV, the antitoxin is a protein that acts as an antagonist to neutralize the action of the protein toxin [35]. In type V, antitoxin is a protein that exhibits site-specific endoribonuclease activity to degrade toxin mRNA, preventing its translation [36]. In type VI, the antitoxin is a proteolytic adaptor protein that neutralizes the protein toxin by promoting its degradation by ClpXP [37]. In type VII, the antitoxin specifically oxidizes the toxin to inactivate it [38].

The type V TA system was proposed based on a study of the GhoT/GhoS system in *E. coli* [36]. This TA system has a protein antitoxin (GhoS) that, under normal growth conditions, exhibits sequence-specific endoribonuclease activity to degrade the toxin (GhoT)-encoded mRNA. However, under conditions of stress, the GhoS-encoding mRNA is degraded by the type II toxin MqsR resulting in the translation of GhoT [39]. Toxin GhoT is a small hydrophobic peptide that damages the cell membrane to reduce adenosine triphosphate and to reduce metabolism in the presence of various antimicrobials related to cell-wall synthesis (that is, carbenicillin and cefoxitin) [9]. A GhoT-like toxin in *E. coli*, named OrtT, was recently described as sharing 63% protein identity with GhoT [10]. Like GhoT, OrtT is a small hydrophobic peptide that damages the cell membrane; however, it lacks a corresponding antitoxin, which makes it an orphan toxin. Furthermore, OrtT acts as an independent toxin to inhibit cell growth and metabolism in the presence of antimicrobials (trimethoprim and sulfamethoxazole) related to amino acid and DNA synthesis [10]. In the present study, we describe the initial characterization of an OrtT homolog in *Salmonella enterica* serovar Houten and two derived peptides that span its two predicted transmembrane regions. We show that the polypeptides are structured as alpha helices when in contact with micelles. In addition, we show that they are toxic, cause membrane damage, and promote cell lysis when overexpressed.

Materials and methods

Computational methods

For the identification of toxins that were homologous to the GhoT/OrtT or GhoS in *S. enterica* serovar Houten, the genome of this bacterium was obtained from a GenBank sequence database (<https://www.ncbi.nlm.nih.gov/>) and it was

analyzed using a method based on profile hidden Markov models (profile-HMM) with the HMMER software suite [40]. Initially, all of the coding sequences of the genome were predicted and translated into protein sequences using PRODIGAL [41]. Each predicted protein was compared to a profile-HMM constructed from a representative sequence alignment of the GhoT/OrtT family (or GhoS family) that was obtained from the Pfam database [42].

Prediction of protein transmembrane domains was performed using the TMHMM server (<http://www.cbs.dtu.dk/services/TMHMM/>). A prediction of the protein structure of the polypeptides was performed with the Phyre2 server [43]. PyMOL (<https://pymol.org/2/>) was used for molecular visualization and three-dimensional image creation. Multiple sequence alignment was created using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). Promoter prediction was performed using the BPROM server (<http://www.softberry.com/>).

Synthesis, purification, and identification of the polypeptides

OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57}, whose sequences are reported in Fig. 2, were chemically synthesized by solid-phase methodology using the Fmoc strategy, which used 0.39 mmol/g of an Fmoc-Phe-Wang resin (for OrtT-Sal and OrtT-Sal_{29–57}) and 0.50 mmol/g of a Rink amide MBHA resin (for OrtT-Sal_{1–29}), and which used diisopropylcarbodiimide (DIC)/N-hydroxybenzotriazole (HOBt) activation [44]. The functional side chains of Fmoc-amino acids were protected by the following groups: (i) tBut (t-Butyl) for Asp, Glu, Ser, and Tyr; (ii) Trt (Trityl) for His, Asn, and Cys; (iii) Pmc for Arg; and (iv) Boc (t-butyloxycarbonyl) for Lys and Trp. Couplings were performed for 2 h at a twofold excess of Fmoc-amino acid and DIC/HOBt reagents in 50% (v/v) methylene chloride (DCM)/dimethylformamide (DMF). The assembly was monitored by the qualitative Kaiser test [45], and if positive, the process was repeated with 50% of reactants. Successive α -amino group deprotection and neutralization steps were performed in 20% 4-methyl-piperidine/DMF for 20 min. After the coupling of the last amino acid was performed, the side chain protecting groups were removed and the resin was cleaved with a solution containing trifluoroacetic acid (TFA) (94.5%), deionized water (2.5%), ethanedithiol (2.5%), and triisopropylsilane (0.5%) at 25 °C for 3 h. Finally, the peptides were precipitated in cold anhydrous ethyl ether, centrifuged, solubilized in 75% (v/v) acetonitrile/H₂O, and lyophilized.

The crude peptides were solubilized in solvent A (0.045% [v/v] TFA/H₂O) and purified by semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu system using a Kromasil C8 column (250 ×

10 mm; 10- μ m particles; 300- $\text{\AA}$ porosity) with a linear gradient of 50–95% (OrtT-Sal), 30–70% (OrtT-Sal_{1–29}), and 20–70% (OrtT-Sal_{29–57}) of solvent B (0.036% [v/v] TFA/acetonitrile) over 90 min. Ultraviolet (UV) detection was carried out at 220 and 280 nm, and the flow rate was 3 mL/min. To estimate the purity of the peptides (> 98%), analytical RP-HPLC was carried out on a UFLC Shimadzu employing a Shimadzu C18 reverse-phase column (250 $\times$ 4.6 mm), using solvents A and B with a linear gradient of 5–95% (v/v) of solvent B for 42 min, at a flow rate of 1.0 mL/min, and with UV detection at 220 and 280 nm. The identity of the peptides was confirmed by electrospray mass spectrometry (Bruker amaZon SL) in positive ionization mode.

Circular dichroism

CD experiments were carried out using a Jasco J-815 spectropolarimeter (JASCO International Co. Ltd.). CD spectra were recorded at room temperature as an average of six scans, using a 1 mm path length quartz cell and spectra in the 190–280-nm range. The secondary structure of the pure polypeptides was estimated in PBS (10 mmol/L phosphate-buffered saline; pH 7.2), 60% 2,2,2-trifluoroethanol (TFE)/PBS, and 10 mmol/L sodium dodecylsulphate (SDS) in PBS. Buffer signals were subtracted in the final spectra of the samples in which TFE or SDS was employed. The peptide concentration was 50 mmol/L.

Preparation of lipid vesicles

Large unilamellar vesicles (LUVs) containing 75% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 22% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and 3% cardiolipin (CL) were prepared by mixing the appropriate lipid amounts in a 4:1 chloroform/methanol mixture in a round-bottom flask. The solvent was evaporated using a rotary evaporator, and then, the flask was placed under vacuum overnight. The lipid film was hydrated in 80 mmol/L 6-carboxyfluorescein (CF) in PBS, and the solution was submitted to six cycles of freezing in liquid nitrogen, thawing at 60 °C, vortexing, and ultrasound. Finally, using an adequate mini extruder (Avanti Lipids Polar Inc.), the suspension was extruded 30 times through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm. CF not incorporated into the vesicles was removed by gel filtration on a Sephadex G-50 column (Sigma-Aldrich Inc.) that was pre-equilibrated in PBS buffer.

Fluorescent dye–release assay

CF release from lipid vesicles was determined by measuring the increase of the intensity of fluorescence after the addition of polypeptides to the system. The acquisition of data was

performed at 25 °C using a Cary Eclipse VARIAN fluorescence spectrophotometer. Wavelengths were set at 492 nm and 517 nm, which corresponded to the excitation and emission wavelengths, respectively, of the CF. Initially, lipid vesicles containing CF were added to the cuvette and upon reaching the first minute of analysis, the polypeptide in the desired concentration was added to the system. At 9 min, the 100% permeabilization value was obtained by the addition of Triton X-100, which caused total vesicle disruption and CF release. The percentage of CF release after the addition of polypeptides to the system was obtained using the equation $[(F_{\text{fin}} - F_{\text{in}})/(F_{\text{max}} - F_{\text{in}})] \times 100$, where F_{in} and F_{fin} represent the initial (before the addition of polypeptides) and final (after the addition of polypeptides) fluorescence values, respectively, and F_{max} represents the fluorescence after the addition of Triton X-100.

Gene synthesis, cloning, and toxicity assay

Coding sequences of OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} (Fig. 2) were synthesized by GenOne (<http://www.genone.com.br>), cloned into a pET24a expression vector (outside of the polyhistidine tag frame), and used to transform *E. coli* Rosetta™(DE3)-competent cells (Novagen). For correct expression of the peptide subunits, a stop codon was added to the end of the OrtT-Sal_{1–29} coding sequence and a methionine codon was added at the start of the OrtT-Sal_{29–57} coding sequence.

For the toxicity assay, overnight cultures of strains producing OrtT-Sal via pET24a-*ortT-Sal*, OrtT-Sal_{1–29} via pET24a-*ortT-Sal*_{1–29}, and OrtT-Sal_{29–57} via pET24a-*ortT-Sal*_{29–57} were inoculated into 50 mL of 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 5.0 g/L NaCl) with kanamycin (50 μ g/mL) to a turbidity at 600 nm of $\sim$ 0.6. Then isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mmol/L, and the turbidity at 600 nm was recorded to determine growth. Three independent cultures were used. All experiments were conducted at 37 °C with slight shaking. For performing the streak toxicity tests, overnight cultures were streaked on 2xYT plates supplemented with kanamycin (50 μ g/mL) and with or without IPTG (0.4 mmol/L) and incubated overnight at 37 °C.

Results and discussion

Theoretical characterization of OrtT-Sal

There are four well-established types of TA modules (types I to IV) that are differentiated by the nature of the antitoxin and by the way in which the antitoxin inhibits the activity of the toxin protein [1]. New TA systems (types V to VII) have been described with regulatory mechanisms that are different from types I to IV

Fig. 1 Theoretical characterization of OrtT-Sal. **a** Multiple sequence alignment between OrtT-Sal, OrtT, and GhoT. **b** Superimposed protein model of OrtT-Sal (blue), OrtT (red), and GhoT (yellow). **c** The chromosomal region around the OrtT-Sal coding sequence. Genic sequences encoding OrtT-Sal (blue arrow), gamma-aminobutyraldehyde

dehydrogenase (yellow arrow), and virulence effector SrfC (green arrow) are indicated. Predicted promoter (σ^H) is shown by the dashed arrow. **d** Chromosomal loci of *ghoT*, *ortT*, and *ghoS* genes into *S. enterica serovar Houten* (GenBank: LS483478.1) and *E. coli* BW25113 (GenBank: CP009273.1) genomes

[36–38]. However, these new systems have been classified on the basis of little available evidence to date. The GhoT/GhoS system is one example of a new TA system; it has been described in *E. coli*, and it represents type V [36]. *E. coli* OrtT is a homologous protein of the membrane-damaging toxin GhoT, but it has been described as an orphan toxin, which means it is not part of a

TA pair [10]. Due to the limited number of studies available on GhoT/OrtT family and because these experimental data are restricted to *E. coli*, we aimed to characterize the biochemical mechanisms of GhoT/OrtT homologs in the related bacteria *S. enterica serovar Houten* in order to confirm and better understand the mechanism of action of these toxins.

Fig. 2 Amino acid sequences of OrtT-Sal and its derived peptides (OrtT-Sal₁₋₂₉ and OrtT-Sal₂₉₋₅₇). The polypeptides were produced by both chemical synthesis and heterologous expression in *E. coli*. In bold is the methionine residue that was added to the OrtT-Sal₂₉₋₅₇ sequence when it

was produced by heterologous expression. In blue and red are cartoon representations of the predicted secondary structure elements (helices) and transmembrane domains, respectively, in the OrtT-Sal protein sequence

Fig. 3 Multiple sequence alignment between GhoS, GhoS-2, and GhoS-Sal

Using a representative sequence alignment of the GhoT/OrtT family (Pfam number PF10753), we constructed a profile hidden Markov model (profile-HMM) that was used to scan the *S. enterica* serovar *Houten* genome (GenBank: LS483478.1) to obtain homologs. From this analysis, only one homologous protein (mentioned in this text as OrtT-Sal) was found. OrtT-Sal, which was the same size as the GhoT and OrtT toxins (57 amino acids), shared 63% protein sequence identity with GhoT and 91% protein sequence identity with OrtT (Fig. 1a). Like GhoT and OrtT, OrtT-Sal was predicted to be a hydrophobic inner-membrane protein with two transmembrane domains (residues 7 to 24 and 34 to 56; Fig.

2). To investigate the structural resemblance of OrtT-Sal with GhoT and OrtT, we modeled these proteins using Phyre2 [43]. The three modeled monomeric proteins had high structural similarities (Fig. 1b). For the three models built, the Phyre2 server used as templates of greater confidence an oligosaccharyltransferase (PDB id 6EZN; an essential membrane protein complex in the endoplasmic reticulum) and a voltage-gated potassium channel protein (PDB id 2P7T; a protein that forms transmembrane channels specific for potassium).

The chromosomal region around the coding sequence of OrtT-Sal was analyzed, and no gene nearby was predicted

Fig. 4 OrtT-Sal is toxic and causes cell lyses when overproduced. **a** Growth of *E. coli* Rosetta™(DE3) cells that harbor an empty plasmid (pET24a) or a plasmid carrying ortT-Sal or a plasmid carrying ortT-Sal₁₋₂₉ or a plasmid carrying ortT-Sal₂₉₋₅₇ in 2xYT plates with kanamycin (50 µg/mL) with or without 0.4 mmol/L IPTG. Three independent cultures were evaluated. **b** Growth of *E. coli* Rosetta™(DE3) cells without a plasmid (pET24a) or harboring an empty plasmid or a plasmid carrying ortT-Sal or a plasmid carrying ortT-Sal₁₋₂₉ or a plasmid carrying ortT-Sal₂₉₋₅₇ in 2xYT medium with kanamycin (50 µg/mL). The black arrow indicates the moment that 0.4 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was introduced. Three independent cultures were evaluated. The error bar represents the standard deviation

encoding antitoxins. In addition, OrtT-Sal was predicted to be under RpoH3 (σ^H) activation (Fig. 1c and Fig. S1). Bacterial sigma factors interact with an RNA polymerase core enzyme and are required to identify the correct sites for transcription initiation. Thus, they are discriminatory, as each binds a distinct set of promoter sequences [46]. RpoH (σ^H or σ^{32}) redirects RNA polymerase to control a subset of genes in a variety of stress environments, maintaining cellular homeostasis [47]. Like OrtT-Sal, OrtT is also predicted to be under RpoH regulation and was shown to be activated in a heat-shock stress environment [10].

As seen, there are some predictions and evidences that indicate the activation of OrtT after stress conditions. However, the mechanism by which this toxin is inactivated after stress remains completely unknown. GhoS antitoxin, of the *ghoS/ghoT* operon, acts by cleaving the *ghoT* transcript [36]. Although OrtT is similar to GhoT, *ortT* mRNA seems not to be a substrate for GhoS [10]. In this context, we also investigated whether the *S. enterica* genome encodes GhoS-like antitoxins. Using the same procedure

employed to search GhoT/OrtT genes, we constructed a profile-HMM from a representative sequence alignment of the GhoS family (Pfam number PF11080), which was used to scan the genome. From this analysis was found only one homologous protein (mentioned in this text as GhoS-Sal) encoded at a position distant $\sim 300,000$ base pairs (bp) from the *ortT-Sal* locus (Fig. 1D). This protein shared 17% protein sequence identity and 39% protein sequence similarity with GhoS (Fig. 3). The genomic sequence of *E. coli* BW25113 strain (GenBank: CP009273.1), which has been used to characterize Ort, GhoT, and GhoS [9, 10, 36], also encodes a GhoS-like protein (mentioned in this text as GhoS-2) that is not in operon with a *ghoT* gene and is localized at $\sim 300,000$ bp from the *ortT* locus (Fig. 1d). However, this protein has not yet been characterized experimentally. GhoS-Sal shared 68% protein sequence identity and 83% protein sequence similarity with GhoS-2 (Fig. 3). Further research is required to assess whether these GhoS-like proteins can degrade *ortT* mRNA.

Fig. 5 Secondary structure of OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} as determined by CD. OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} were analyzed by circular dichroism to determine their secondary structure. Each polypeptide was evaluated in three environments: PBS buffer only, TFE in PBS, and SDS in PBS. All polypeptides only displayed α -helical structure in the presence of TFE or SDS micelles

OrtT-Sal is toxic and may cause cell lysis when overproduced

Because GhoT and OrtT cause membrane damage, their overproduction results in severe growth inhibition and cell lysis [10, 36]. To investigate whether this also occurs with OrtT-Sal, we produced OrtT-Sal in an *E. coli* expression system that served as a bacterial membrane model for in vivo overexpression studies. Moreover, we also produced its N- and C-terminal regions, which span their predicted transmembrane domains (OrtT-Sal_{1–29} and OrtT-Sal_{29–57}, respectively; Fig. 2). We found that overproduction of OrtT-Sal, OrtT-Sal_{1–29}, and

OrtT-Sal_{29–57} caused growth inhibition (Fig. 4a). Corroborating these results, overproduction of these polypeptides caused a decrease in the turbidity of the culture (Fig. 4b), suggesting cell lysis. Similar results have been reported for GhoT and OrtT when overexpressed [10, 36].

Conformational changes of OrtT-Sal in SDS micelles and in TFE

Because OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} are toxic and to enable in vitro studies of conformational changes, the polypeptides (Fig. 2) were chemically synthesized by solid-phase and

Fig. 6 Fluorescent dye-release assay. **a** OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} increase the release of CF from lipid vesicles based on their concentrations. CF release from lipid vesicles was determined by measuring the increase of the intensity of fluorescence after the addition of polypeptides, at first minute, in the desired concentration. The control

represents the absence of polypeptides (addition of PBS buffer only). At 9 min, Triton X-100 was added to yield the 100% permeabilization and to allow the normalization of the values of CF release. **b** CF released by OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57}

subsequently purified. The secondary structure of the polypeptides was analyzed by circular dichroism (CD) spectroscopy in phosphate-buffered saline (PBS) and in the presence of sodium dodecylsulphate (SDS), which created a membrane-mimetic environment that mimicked the negative charges of the bacterial membranes [48]. Furthermore, the structural conformation of the synthetic peptides was studied in the presence of trifluoroethanol (TFE), a solvent that induces and stabilizes α -helical structures in polypeptides with intrinsic tendencies to adopt this kind of secondary structure in solution [49, 50]. No secondary structures were observed in the absence of SDS and TFE; in the presence of SDS or TFE, CD spectrum of all polypeptides displayed α -helix conformational structures, showing a negative peak around 222 nm and 208 nm and a positive peak around 195 nm (Fig. 5). This secondary structuration dependent of the micellar environment was also observed for GhoT when in contact with liposomes [12].

Lipid vesicles damaged by OrtT-Sal

To study if the synthetic polypeptides OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} (Fig. 2) cause damage to membranes, an in vitro fluorescent dye-release assay was performed. As carboxyfluorescein (CF) is a self-quenching fluorophore, with fluorescence measurable only at low concentrations [51], helical peptides that disrupt the lipid membranes either by forming pores or by complete micellization can be characterized by CF release from lipid vesicles. In this context, the permeabilization of lipid vesicles was evidenced by an increase in the intensity of fluorescence after the addition of polypeptides to the system. Unilamellar vesicles containing 75% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 22% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and 3% cardiolipin (CL) were employed as a model of the inner bacterial membrane [52, 53]. Our findings indicated that OrtT-Sal, OrtT-Sal_{1–29}, and OrtT-Sal_{29–57} caused damage to the lipid vesicles, and the CF release increased with the increase of the peptide concentration (Fig. 6a). OrtT-Sal and OrtT-Sal_{1–29} were able to permeabilize 100% of the lipid vesicles in 9 min at the highest concentration used (that is, 2.5 μ mol/L of polypeptides). This direct membrane damage activity was also observed for GhoT when in contact with liposomes [12]. In addition, corroborating the results from the in vivo toxicity assay (Fig. 4a, b), OrtT-Sal_{1–29} showed greater membrane damage capacity than the other polypeptides, and the OrtT-Sal_{29–57} showed the lowest activity between them (Fig. 6a, b). The CD spectrum for OrtT-Sal_{1–29} shows that this subunit has a more stable structuration relative to the other polypeptides, since it presented higher signal intensity (Fig. 5). This fact may have been determinant for OrtT-Sal_{1–29} to exhibit greater membrane damage activity. The same reasoning can be used for the OrtT-Sal_{29–57} subunit, which presented the worse structuration and the lowest activity.

Conclusions

Our findings contributed to the confirmation that the OrtT-Sal toxin found in *S. enterica* serovar Houten is a small hydrophobic protein that damages lipid membranes. Moreover, the structural studies performed here prove for the first time that OrtT-Sal has two transmembrane domains that are structured as alpha helices, and that the secondary structures are dependent upon contact with the membrane environment. Further research is required to confirm if this homolog in *S. enterica* serovar Houten is activated by sigma factor RpoH and to evaluate its physiological role and how it is inactivated after stress.

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