



## New insights about the EptA protein and its correlation with the *pmrC* gene in polymyxin resistance in *Pseudomonas aeruginosa*

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### ABSTRACT

Nowadays, clinical and scientific interest in antibiotics, as polymyxin, has increased due to the large number of reports of multiresistant Gram-negative bacteria, as *Pseudomonas aeruginosa*. The aim of this study was to investigate a related group of proteins for resistance to polymyxins, encoded by *P. aeruginosa* genome, through *in silico* analysis. The mobilized colistin resistance 1 (MCR<sub>1</sub>) protein from *Escherichia coli* was used for comparison. Similar sequences to the protein MCR<sub>1</sub> in *P. aeruginosa* were analysed for physicochemical properties. 31 protein isoforms in *P. aeruginosa* (EptA) were found able to confer resistance to polymyxin showing protein lengths between 551 and 572 amino acids, with molecular mass values between 61.36 - 62.80 kDa, isoelectric point between 6.10 to 7.17, instability index between 33.76 to 41.87, aliphatic index between 98.67 to 102.63 and the hydropathy index between -0.008 to 0.094. These proteins belong to the DUF<sub>1705</sub> superfamily with bit-score values between 559.81 and 629.78. A high degree of similarity between EptAs in *P. aeruginosa* was observed in relation to other proteins that confer resistance to polymyxins, present in Gram-negative bacteria species of clinical interest. Although, further studies are needed to identify the actual contribution of EptAs in *P. aeruginosa* species.

### Introduction

Polymyxins are classified into five groups, named: A, B, C, D and E (Neiva et al., 2013). Although, currently only polymyxins B and E are commercially available (Pogue et al., 2017), or used in clinical interventions (Lorenzo et al., 2011), due to high toxicity of the other isoforms (Falagas and Kasiakou, 2005). This group of antibiotics was discovered in 1947 (Li et al., 2005), and polymyxin E was the first to be used in the clinic, in 1959 (Yu et al., 2015). Polymyxins were identified in *Bacillus polymyxa* strains (Stansly and Schlosser, 1947; Girardello and Gales, 2012), subspecies: *colistin* *Koyamae* (Lorenzo et al., 2011). Polymyxins B and E have potent antimicrobial activity against several species of Gram-negative bacteria (Mendes and Burdmann, 2009; Carvalho and Cogo, 2014). Some hypotheses could explain the mechanism of polymyxins resistance acquired by *P. aeruginosa*: (I) Adaptive mechanism: Gradual adaptation, due to the presence of this antimicrobial

component associated with the culture medium used in experimental settings or in clinical diagnosis (Zavascki et al., 2010).

Another possibility is the interference of this drug during active transport through the membrane, specifically in the lipid A portion of bacterial lipopolysaccharides (LPS) (Mendes and Burdmann, 2009) resulting in loss of OMPs (Outer Membrane Proteins) or reduction of interactions between polymyxin and the envelope (Moore et al., 1984); (II) Genetic mutation mechanism: Associated with increased levels of H<sub>1</sub> protein (H<sub>1</sub>-T6SS), and cations replacement, minimizing the Mg<sup>2+</sup> concentration and increasing the Ca<sup>2+</sup> concentration in cell membrane, reducing possible electrostatic interactions with polymyxin (Zhang et al., 2011; Fair and Tor, 2014; Morita et al., 2014). In 2010, it was shown that H<sub>1</sub>-T6SS in *P. aeruginosa* has three effector proteins, called Tse<sub>1-3</sub> (type VI secretion exporters 1–3), where Tse<sub>1</sub> and Tse<sub>3</sub> can cleave peptidoglycan associated with the bacterial envelope (Hood et al., 2010), and this function of H<sub>1</sub>-T6SS is directly linked to antibiotic

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resistance in biofilms (Zhang et al., 2011).

Furthermore, absence of 2-hydroxy-laureate and presence of 4-aminoarabinose in bacteria membrane and an increased A palmitate concentration in lipid A (Bonomo and Szabo, 2006), besides mutations in the PhoPQ and PmrAB systems that induces modified LPS operon (*arm*), resulting in addition to Lara4N (4-amino-4-deoxy-L-arabinose) in the lipid A portion of the LPS, could potentialize the resistance to polymyxins in *P. aeruginosa* (Schurek, 2009). Different modifications in lipid A may occur, including changes in the number of acyl chains and phosphoethanolamine addition (Tran et al., 2005; Herrera et al., 2010). Other possible resistance factors to polymyxins could be the induction of the *pmrCAB* operon. These genes comprise a three-component system: response regulator (*pmrA*), histidine kinase sensor (*pmrB*), and the protein that adds phosphoethanolamine to lipid A (*pmrC*).

Thus, polymyxins resistance could be related to increased expression of *pmrC* gene. In *P. aeruginosa*, *N. meningitidis*, *S. enterica* and *S. plymuthica* the product of the *pmrC* gene it is known as EptAs, whereas in the bacterium *Escherichia coli* it is known as MCR<sub>1</sub>, and *Klebsiella pneumoniae* it is known as MCR<sub>1.9</sub>. In this context, *P. aeruginosa*, a Gram-negative bacterium, in the last decade has received much attention due to its capacity to cause serious infections in Brazilian hospitals (Lopes, 2009; Silva Filho and Silva, 2013; Bomfim and Knob, 2013). For this reason, this work aims to study the physicochemical properties of EptA proteins expressed in *P. aeruginosa* genomes deposited at the National Center for Biotechnology Information - NCBI (<https://www.ncbi.nlm.nih.gov/>) database, to improve the understanding of the mechanisms of resistance to polymyxins developed by this bacterial species.

## Methods

### Obtaining and analysing gene sequences

In this experimental approach, the National Center for Biotechnology Information - NCBI (<https://www.ncbi.nlm.nih.gov/>) database was initially evaluated to identify clusters that encode isoforms of the enzyme Lipid A phosphoethanolamine transferase (EptA) in *P. aeruginosa* genome. For this purpose, the EptA sequence (product of the *mcr1* gene of *E. coli*, Access in GenBank: ASK04346.1) was used as bait to obtain target protein sequences from the *Protein Blast tool* (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Physicochemical characterization

To physicochemically characterize the EptAs isoforms present in different *P. aeruginosa* genomes we used: ProtParam server - ExPasy for analysis of theoretical molecular weight (rM), isoelectric point (pI), instability index (II), aliphatic index (IA) and the grand average of hydropathicity index (GRAVY) (<https://web.expasy.org/protparam/>).

### Prediction of secondary structure and protein domain

Secondary structures of the EptA isoforms were predicted using the SopMa - Secondary Structure Prediction Method server (Geourjon and Deléage, 1995). For protein domain prediction all .fasta extension sequences were submitted to Conserved Domains Database (CDD) and Resources - NCBI - NIH server (Lu, 2020).

### Phylogenetic analysis

The phylogenetic analysis was obtained from the alignment of EptA from *P. aeruginosa* sequences. For determination of the phylogenetic tree, the Neighbor Joining method (NJ) was used with pairwise deletion option and reliability index of 1000 replicas of bootstrap. The analysis was performed in the MEGA X Molecular Evolutionary Genetics Analysis - Phylogenetic (Kumar et al., 2018) software.

## Results and discussions

*P. aeruginosa* is classified as a Gram-negative bacterium and are very versatile, capable of spreading on soils (Al-Saleh and Akbar, 2015), marshes (Teixeira et al., 2016), coastal marine habitats (Habbu et al., 2016), as well as plants and animals (Ellison et al., 2013). In this context, the easy environmental adaptation and dissemination have aroused the curiosity of many researchers around the world, resulting in several *P. aeruginosa* genomes being sequenced (genomes data were obtained from the National Center for Biotechnology Information - NCBI (<https://www.ncbi.nlm.nih.gov/>)). The microorganism was found in many researches conducted in intensive care units (ICUs) (Basso et al., 2016). In Belém-PA, Brazil, infections caused by *P. aeruginosa* resulted in the second highest mortality rate in ICUs, only behind *Mycobacterium tuberculosis* species (Barros et al., 2016). Polymyxins B and E (colistin) are antibiotics that *P. aeruginosa* developed resistance against (Lorenzo et al., 2011; Barros et al., 2016; Lee et al., 2012), although the mechanism leading to it is not very clear (Stefani et al., 2017; Gutu, 2013).

On the other hand, in microorganisms as *Acinetobacter baumannii*, *E. coli* and *Klebsiella pneumoniae* this mechanism is well known, being a direct result of the mobile colistin resistance *mcr1* gene (Olaitan et al., 2014; Chang et al., 2012; Liu, 2016). It is a common sense that the emergence of polymyxin-resistant strains could be associated with the use of colistin in agriculture and animal farms (Zheng et al., 2018; Economou and Gousia, 2015; Nguyen et al., 2016; Zheng et al., 2018). The *Protein Blast server* (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was searched for sequences related to the *mcr1* gene of *E. coli* present in genomes belonging to *Pseudomonas* genus. 101 protein sequences were identified, of which 80 were associated with *P. aeruginosa*, 4 with *P. citronnellolis*, 4 with *P. nitroreducens*, 2 with *P. alcaligenes*, 2 with *P. jinjuensis*, and 9 other species, with all sequences belonging to *Pseudomonas* genus. Only the isoforms catalogued for the *P. aeruginosa* species were analysed, being distributed currently, among 18 known genomes, according to the identification of the rates (Table 1).

To avoid redundant data, all 80 EptA sequences from *P. aeruginosa* species were subjected to identity matrix analysis using the MEGA X software (Teixeira et al., 2016) resulting in only 59 unique sequences. Then, these sequences, were evaluated regarding the protein domain that they belong. After this evaluation, only 31 EptA isoforms remained, all belonging to the DUF<sub>1705</sub> superfamily, similar to proteins encoded by *E. coli* (Stoesser et al., 2016), *Salmonella enterica* (Doumith, 2016), *K. pneumoniae* (Di Pilato et al., 2016), *Serratia plymuthica* strain AS<sub>13</sub> e *Neisseria meningitidis* (Zhang et al., 2019), all Gram-negative bacteria. Some members of this superfamily are putative bacterial membrane proteins.

The number of amino acids presents in EptA isoforms of *P. aeruginosa* ranged from 551 to 572 amino acids (aa), and it was possible to recognize three groups based on the sequence size: (I) eleven isoforms

**Table 1**

List of Gram-negative bacteria, *Pseudomonas aeruginosa* genomes, deposited at the National Center for Biotechnology Information - NCBI (<https://www.ncbi.nlm.nih.gov/>).

<i>Pseudomonas aeruginosa</i> strains	
<i>P. aeruginosa</i> (taxid:287)	<i>P. aeruginosa</i> OS <sub>42</sub> (taxid:1402581)
<i>P. aeruginosa</i> group (taxid:136841)	<i>P. aeruginosa</i> BWHPA <sub>028</sub> (taxid:1402528)
<i>P. aeruginosa</i> PAO <sub>1</sub> (taxid:208964)	<i>P. aeruginosa</i> BL <sub>12</sub> (taxid:1402553)
<i>P. aeruginosa</i> PAK (taxid:1009714)	<i>P. aeruginosa</i> PA <sub>7</sub> (taxid:381754)
<i>P. aeruginosa</i> DSM 50071 (taxid:1123015)	<i>P. aeruginosa</i> BL <sub>04</sub> (taxid:1402545)
<i>P. aeruginosa</i> E <sub>2</sub> (taxid:1163395)	<i>P. aeruginosa</i> ATCC <sub>25324</sub> (taxid:1163393)
<i>P. aeruginosa</i> str. PA <sub>17</sub> (taxid:1333546)	<i>P. aeruginosa</i> VRFPA <sub>07</sub> (taxid:1431713)
<i>P. aeruginosa</i> PA <sub>38182</sub> (taxid:1407059)	<i>P. aeruginosa</i> UCPPP-PA <sub>14</sub> (taxid:208963)
<i>P. aeruginosa</i> str. Stone <sub>130</sub> (taxid:1125697)	<i>P. aeruginosa</i> BWHPA <sub>037</sub> (taxid:1402529)

formed by 551 aa; (II) a single isoform formed by 567 aa; and (III) two isoforms formed by 572 aa in length. The results obtained in this work in relation to the size of the sequences in *P. aeruginosa* EptA enzymes showed that it is larger than other proteins from *E. coli* MCR<sub>1</sub>, *K. pneumoniae* MCR<sub>1,9</sub> and *S. enterica* MCR<sub>1,6</sub> (GenBank ids: ASK04346.1, ARA74236.1 and WP\_077248208.1, respectively) which has 541 aa (Compain et al., 2014), *N. meningitidis* EptA (GenBank id: ELL31702.1) which has 544 aa, and *S. plymuthica* EptA (GenBank id: KYG15235.1) which has 545 aa.

These results could be due to sequencing processes, or even location of EptA gene in a region of the *P. aeruginosa* genome difficult to sequence. These enzymes are still in process of evolution since the reports of polymyxins resistance in *P. aeruginosa* are very recent (Olaitan et al., 2014) for this reason the EptA isoform could currently have a smaller length than other microorganisms. The most frequent amino acids in *P. aeruginosa* EptAs belongs to the group of the aliphatic amino acids, including: leucine (13.6% to 17.1%), alanine (7.6% to 11.4%), glycine (6.7% to 7.9%), valine (5.2% to 7.8%) and the polar uncharged serine (5.8% to 6.6%), respectively. Moreover, amino acids in lower frequency are: the aromatic amino acid tryptophan (1.3% to 1.6%), the aliphatic amino acid methionine (1.7% to 2.2%), the polar uncharged amino acid cysteine (1.9% to 2.2%), and the positively charged amino acid histidine (1.7% to 2.4%).

When the EptAs in *P. aeruginosa* were compared with other microorganisms, a pattern of higher and lower frequency of amino acids was found, suggesting that these isoforms were derived from a single gene. When compared to the proteins MCR<sub>1</sub> of *E. coli*, MCR<sub>1,9</sub> of *K. pneumoniae*, and EptAs of *S. enterica*, *N. meningitidis* and *S. plymuthica* the same pattern repetition in relation amino acids frequency was found, with high rates of leucines, valines, alanines and serines. Only in the

EptA isoforms from *P. aeruginosa* the glycine residues are among the five most frequent amino acids, being replaced by threonine (uncharged amino acid). In relation to the less frequent amino acids: methionine, cysteine and histidine, there is also repetition in the EptAs of these microorganisms (*E. coli*, *K. pneumoniae*, *N. meningitidis*, *S. enterica*, and *S. plymuthica*).

Only phenylalanine the fifth most frequent amino acid in EptA isoforms of *P. aeruginosa*, is replaced by tyrosine in the EptA of *S. plymuthica*, while the methionine amino acid, second most frequent in *P. aeruginosa* EptA, is replaced by glycine. Few differences in frequency of each amino acid may be directly associated with the different lengths of polypeptide chains that form these proteins in these microorganisms. The molecular mass (average) predicted for EptA isoforms of *P. aeruginosa*, showed variations from 61.36 to 62.80 kDa (Table 2). In this analysis two generic groups were observed based on their molecular masses (I) formed by 10 isoforms (32.26%, molecular mass approximately 61.5 kDa); (II) formed by 21 isoforms (67.74%, molecular mass approximately 62.5 kDa). In relation to the protein molecular masses of *E. coli* MCR<sub>1</sub> (60.12 kDa), *K. pneumoniae* MCR<sub>1,9</sub> (60.09 kDa) and EptAs of *S. enterica* (60.10 kDa), *N. meningitidis* (61.36 kDa) and *S. plymuthica* (61.64 kDa). This result may be due to the same reasons discussed above about the lengths of these proteins sequences. Although all these EptAs proteins belong to the DUF<sub>1705</sub> superfamily (Lu, 2020), the isoforms expressed in *P. aeruginosa*, classified in group (I), are likely to be more active against polymyxins B and E, because their size is closer to the EptA of *E. coli* (Stoesser et al., 2016), *S. enterica* (Doumith, 2016), *K. pneumoniae* (Di Pilato et al., 2016), *S. plymuthica* AS<sub>13</sub> strain.

The concentration of hydrogen ions in a solution where the ionization of the acid groups are equal to the ionization of the basic groups is known as the isoelectric point (Highberger, 1939). Data obtained on the

**Table 2**

Identification of EptAs amino acid sequences encoded by the bacterium *Pseudomonas aeruginosa* based on access from the National Center for Biotechnology Information - NCBI (<https://www.ncbi.nlm.nih.gov/>). Domain analysis: Position-Specific Scoring Matrix (PSSM id), Domain (starting and ending amino acid), and Bit-score E-value were obtained after access to the Conserved Domain Database - CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). Physicochemical analysis: Molecular weight (rM), Theoretical (pI), Instability index (II), Aliphatic index (IA), and Grand average of hydropathicity (GRAVY) were obtained after access to the ProtParam tool (<https://web.expasy.org/protparam/>). All data revisited and checked on April 20th, 2021.

GenBank id	Size (aa)	Domain		Bit-score	rM (kDa)	pI	II	IA	GRAVY
		Start	end						
OKO10373.1	565	5	545	559.81	61.43	6.89	34.99	101.23	0.085
WP_012076057.1	588	13	538	629.78	62.80	6.57	41.54	99.35	0.001
WP_019485721.1	565	5	545	594.73	61.43	6.89	34.50	101.23	0.085
WP_023081504.1	565	5	545	594.73	61.40	6.89	34.85	101.05	0.084
WP_023118443.1	583	13	538	616.69	62.38	6.24	40.89	101.92	0.026
WP_023120612.1	583	13	538	616.30	62.36	6.39	40.44	102.28	0.039
WP_024914867.1	565	5	545	595.50	61.42	6.54	33.76	100.69	0.082
WP_031694049.1	583	13	538	615.92	62.32	6.24	39.73	101.94	0.035
WP_033962259.1	583	13	538	617.07	62.41	6.24	40.41	102.10	0.030
WP_033981880.1	565	5	545	593.58	61.43	6.89	35.65	101.23	0.085
WP_033997080.1	588	13	538	615.15	62.77	6.57	41.87	99.53	0.006
WP_034015152.1	583	13	538	629.78	62.35	6.39	40.99	101.76	0.035
WP_034038915.1	583	13	538	615.92	62.36	6.10	40.29	101.59	0.022
WP_034053930.1	583	13	538	614.76	62.41	6.24	41.31	101.76	0.035
WP_034070146.1	583	13	539	615.15	62.35	6.24	40.60	102.63	0.040
WP_034072350.1	583	13	538	619.77	62.35	6.39	40.22	102.45	0.048
WP_034075934.1	583	13	538	616.69	62.44	6.24	40.15	101.92	0.025
WP_034082976.1	588	13	538	615.53	62.76	6.57	41.87	98.85	-0.003
WP_043089441.1	583	13	538	616.69	62.49	6.10	40.30	101.92	0.020
WP_044061861.1	565	5	545	595.89	61.45	6.89	34.85	101.48	0.089
WP_054379545.1	583	13	539	616.69	62.34	6.24	40.94	102.45	0.043
WP_057393034.1	565	5	545	593.96	61.42	7.17	33.76	101.05	0.083
WP_058135045.1	588	13	538	627.86	62.78	6.57	41.87	98.67	-0.008
WP_058178590.1	583	13	538	617.84	62.33	6.24	40.56	101.94	0.035
WP_083556735.1	565	5	545	601.28	61.51	7.17	35.20	101.40	0.082
WP_086340251.1	565	5	545	593.58	61.38	6.89	34.70	100.53	0.076
WP_086822489.1	583	13	538	616.69	62.35	6.10	41.84	101.76	0.030
WP_088171235.1	565	5	545	595.12	61.43	6.89	34.50	101.76	0.094
WP_093945147.1	583	13	538	616.69	62.35	6.39	40.40	101.41	0.028
WP_096229894.1	565	5	592	592.81	61.36	7.17	34.35	101.05	0.090
WP_096262676.1	565	5	545	594.35	61.42	7.17	35.30	101.05	0.084

isoelectric point (pI) of EptA isoforms of the microorganism *P. aeruginosa* demonstrated that 27 isoforms are acidic and only four are basic. Analyses performed with the proteins of other species demonstrated a pI of 6.31 for *E. coli* MCR<sub>1</sub>, and *K. pneumoniae* MCR<sub>1,9</sub>, 6.23 for *S. enterica* EptA, 6.67 for *S. plymuthica* EptA, that is, isoforms with acid pI, whereas the EptA belonging in *N. meningitidis* species feature pI of 7.57, that is, a pI basic. This observation is in perfect agreement with the EptA isoforms from *P. aeruginosa*, since 87.10% showed acidic pI and only 12.90% showed basic pI. This variation in isoelectric points presented by EptAs expressed in *P. aeruginosa* may be directly associated to its adaptive potential in different environments (Kumar et al., 2018; Teixeira et al., 2016; Al-Saleh and Akbar, 2015).

The instability index provides an estimate of how much a protein can remain stable during an experiment conducted in a test tube (Gurusprasad et al., 1990). The instability index provides an inverse estimate of the metabolic stability of a protein (<https://web.expasy.org/protaram/>). All EptAs in *P. aeruginosa* sequences analysed in this study predicted instability index values ranging from 33.76 to 41.87, so all these isoforms are relatively stable analysis in test tubes (Table 2). The analysis of the instability index indicated values of 22.65 for *E. coli* MCR<sub>1</sub> and *S. enterica* EptA protein (Doumith, 2016; Di Pilato et al., 2016), 21.78 for *K. pneumoniae* MCR<sub>1,9</sub> (Zhang et al., 2019), 35.77 for *N. meningitidis* EptA and 41.11 for *S. plymuthica* EptA. Based on observed rates for *P. aeruginosa* EptA, the proteins from *E. coli* MCR<sub>1</sub>, *K. pneumoniae* MCR<sub>1,9</sub>, *S. enterica* and *N. meningitidis* EptAs are stable. The *S. plymuthica* species was the only one that presented unstable EptA. Similarly, in *P. aeruginosa*, two groups EptA were observed, the first group formed by 18 stable proteins (58.06%), and second by 13 unstable proteins (41.94%).

The protein aliphatic index is defined as the relative volume occupied by aliphatic side chains: alanine, valine, isoleucine and leucine (Ikai, 1980). It is generally considered a positive factor to increase protein thermostability, therefore, it demonstrates how much protein molecules are able to withstand heat denaturation. The results obtained on thermostability for the EptA isoforms of *P. aeruginosa* have shown values between 98.67 and 102.63 corroborating the previous data, indicating that many of these proteins are really stable (Ikai, 1980). When the aliphatic index of the other proteins was evaluated it presented values of 92.83 for *E. coli* MCR<sub>1</sub> and *S. enterica* EptA, 93.03 for *S. plymuthica* EptA, 93.36 for *K. pneumoniae* MCR<sub>1,9</sub>, and 96.21 for *N. meningitidis* EptA. Based on rates observed for *P. aeruginosa* EptA, the majority of the proteins are stable at 25 °C temperature as presented in the species *E. coli*, *K. pneumoniae*, *S. enterica*, and *N. meningitidis*. It is possible that *P. aeruginosa* EptA can tolerate higher temperatures because these proteins are important both in environmental adaptation as well *in vivo* febrile hosts infection (Ikai, 1980; Zhang et al., 2012; Chan et al., 2016).

The GRAVY value (hydrophobicity index) for a peptide or protein is calculated based on the sum of all amino acids hydropathy values, divided by the total number of residues present in the sequence (Oliveira et al., 2020). High positive values means that the amino acids located in the protein region are more hydrophobic. These scales are commonly used to predict the transmembrane alpha helices of membrane proteins (Kyte and Doolittle, 1982). GRAVY analysis from *P. aeruginosa* EptAs profile revealed that 29 isoforms (93.55%) are hydrophobic and 2 (6.45%) are hydrophilic (Table 2). This result indicates that mostly of these enzymes may be in transmembrane regions in the *P. aeruginosa* bacteria, while the other group of these enzymes must have differentiated their metabolic function. In other bacterial species, the evaluation of GRAVY indicated a value of 0,067 for *S. enterica* EptA, 0, 018 for *N. meningitidis* EptA, 0,065 for *E. coli* MCR<sub>1</sub>, 0,067 for *S. enterica* EptA and 0,069 for *K. pneumoniae* MCR<sub>1,9</sub>, all with hydrophobic profile.

Although, the hydrophilic *P. aeruginosa* EptAs role is not known, they are very similar to LptB protein, when the amino acid sequences are analysed, both are soluble proteins without transmembrane domain. According to Sperandeo et al., LptB was identified in an internal

membrane complex in *E. coli*, forming a 140 kDa protein agglomerate, although no other proteins were detected (Sperandeo, 2007; Stenberg et al., 2005).

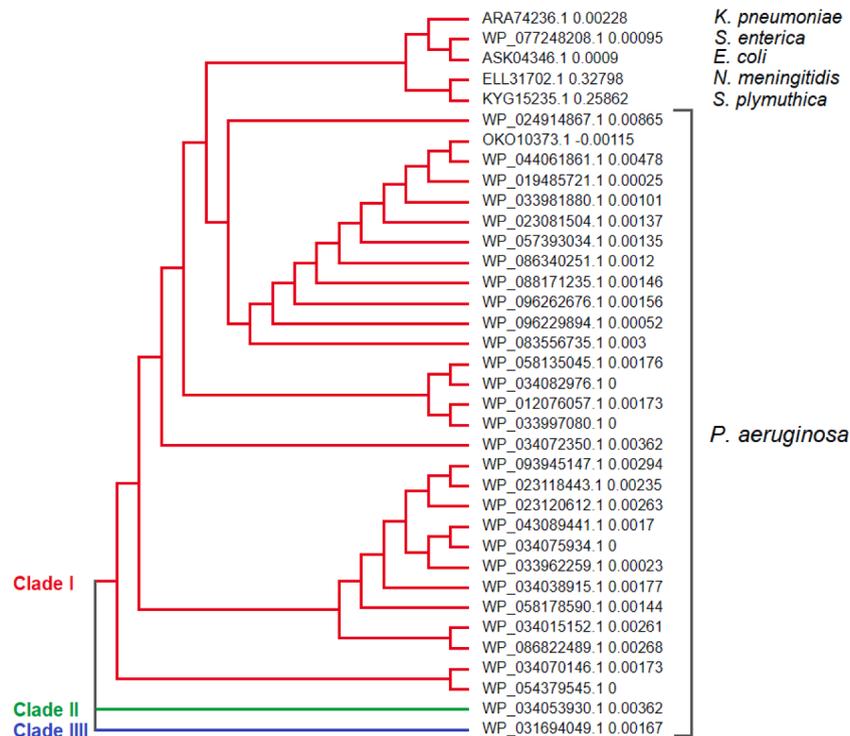
We suggest that hydrophobic and hydrophilic EptA in *P. aeruginosa* may interact in a similar way like LptA and LptB in *E. coli* and *K. pneumoniae*, together with a transmembrane partner unidentified yet, resulting in a membrane-associated complex required to transport deleterious molecules to this microorganism. On the other hand, the bacterial LPS modification, with the cations substitution present in the phosphate groups by L-Ara4N, neutralizing the lipids A charges, or PEtN modification, increases the net load from -1.5 to -1 (Nikaido, 2003). The L-Ara4N modification is the most effective of the two modifications due to the nature of the charge change. The net positive charge resulting from the modified LPS reduces its binding to polymyxins, leading to resistance (Olaitan et al., 2014). The composition of amino acid residues is directly associated with the structure and protein functions.

The secondary structures predicted for EptAs in *P. aeruginosa* using the SopMA server showed possible data grouped into two subgroups: (I) consisting of 20 isoforms (64.51%) with predominance of  $\alpha$ -helices (44.15 - 47.68%), followed by loops (28.76 - 30.31%) and  $\beta$ -sheets (22.58 - 25.72%); (II) formed by 11 isoforms (35.49%) with predominance of loops (39.35 - 41.51%), followed by  $\alpha$ -helices (30.75 - 34.18%) and  $\beta$ -sheets (26,12–28, 61%). When comparing the data obtained for EptAs from *P. aeruginosa* with MCR<sub>1</sub> in *E. coli*, MCR<sub>1,9</sub> in *K. pneumoniae* and EptAs in *S. enterica*, *N. meningitidis* and *S. plymuthica*, there is a greater proximity to the EptAs belonging to subgroup (I), since  $\alpha$ -helices are predominant, followed by loops and  $\beta$ -sheets.

Although such differences can be observed in *P. aeruginosa* EptAs, this phenomenon could be explained due to variations observed in the polypeptides sequences, which could be associated with the thermostability shown by this isoforms (Ikai, 1980; Zhang et al., 2012), considering the combination of selective pressure factors that have acted on these proteins for decades (pressure, pH, temperature), besides the mutations that have accumulated. According to the analysis performed using Conserved Domains Database - CDD (Lu, 2020), all EptAs expressed by *P. aeruginosa* belong to the superfamily DUF<sub>1705</sub> majority members of this family are putative bacterial membrane proteins, and this domain is found immediately in the N-terminal region, likewise all those used in this work as standard, included, MCR<sub>1</sub> of *E. coli*, MCR<sub>1,9</sub> of *K. pneumoniae* and EptAs of *S. enterica*, *N. meningitidis* and *S. plymuthica*.

The alignment of the EptAs sequences was performed using the Molecular Evolutionary Genetics Analysis - MEGA X software. It revealed low amino acid variability along the polypeptide chains, however, these substitutions are equivalent, what means they belong to the same functional group (Data not shown). The largest consensus area is located between the amino acids 240 until the C-terminal region of these proteins. It is also possible to observe that the N-terminal region has a higher variation in these proteins. The dendrogram was generated with support of the MEGA X software (Teixeira et al., 2016), with the aid of sequence alignment previously performed. It was shown that EptAs from *P. aeruginosa* formed two distinct clades as can be seen in Fig. 1: Clade I concentrates 93.55% of the identified isoforms. Interestingly, the MCR<sub>1</sub> proteins of *E. coli*, MCR<sub>1,9</sub> of *K. pneumoniae* and EptA of *S. enterica* were organized in the clade I, such result corroborates with the other results previously presented, strengthening other findings that predict the participation of these proteins in the mechanism of the resistance to polymyxins by members from *Enterobacteriaceae* family (Nikaido, 2003; Alves and Behar, 2013; Andino and Hanning, 2015). In relation to, the clades II and III, both are formed by only one isoform (WP\_034053930.1 and WP\_031694049.1, respectively).

Although the *S. plymuthica* species are classified as an *Enterobacteriaceae*, in clinical settings, members belonging to the *Serratia* genus, generally cause infections in the respiratory system (Domingo et al., 1994), urinary tract (Jain et al., 2017) and can cause necrotic cellulitis (Mahlen, 2011), instead of infections in the gastrointestinal tract, as observed with other microorganisms in this family. In addition, small



**Fig. 1.** Cladogram generated by the Neighbor-Joining method employing alignment and multiple performance with Molecular Evolutionary Genetics Analysis software - MEGA X. Proteins belonging to the DUF<sub>1705</sub> superfamily of *P. aeruginosa* and proteins responsible for polymyxin resistance in Gram-negative bacteria with clinical interest. Node values = bootstrap test (1000 pseudo-replicas).

mammals, vegetables and water seem to be the natural environment of *S. plymuthica* species (Grimont and Grimont, 2006). These *S. plymuthica* characteristics may be the cause of their differentiated grouping in relation to other enterobacteria as *E. coli*, *K. pneumoniae* and *S. enterica*. In the same way as *S. plymuthica*, *N. meningitidis*, was classified in a group of its own, because this species presents different biology among the other species discussed in this study. *N. meningitidis* is a Gram-negative *Diplococcus* and humans are the only natural host (Souza and Gagliani, 2011), causing cerebrospinal meningitis and/or septicaemia (Coureuil et al., 2013).

Considering the cladogram, it is possible to identify the way of acquisition of antimicrobial resistance to polymyxins. *P. aeruginosa* appears to be the species that initially developed resistance to polymyxins, followed by enterobacteria *K. pneumoniae*, *E. coli* and *S. enterica*, and then *N. meningitidis* and *S. plymuthica* bacteria. Finally, *P. aeruginosa* through EtpA proteins began to resist the action of polymyxins.

## Conclusion

In conclusion these results showed the high degree of similarity between EptA proteins of *P. aeruginosa* with MCR<sub>1</sub> of *E. coli*, MCR<sub>1,9</sub> of *K. pneumoniae* and EptA proteins of *S. enterica*, *N. meningitidis* and *S. plymuthica*, based on their physicochemical properties, we demonstrated 31 protein isoforms in *P. aeruginosa* (EptA) found in to 18 genomes from *P. aeruginosa* closely related to proteins known to provide resistance to polymyxin in other species of Gram-negative bacteria of clinical interest. EptAs from *P. aeruginosa* studied here, belonging to DUF<sub>1705</sub> family as indicated by values bit-score between 559.81 and 629.78. In addition, these EptAs showing lengths between 551 and 572 amino acids, molecular mass values between 61.36 - 62. 80 kDa, isoelectric point between 6.10 to 7.17, instability index between 33.76 to 41.87, aliphatic index between 98.67 to 102.63 and the hydropathy index between - 0.008 to 0.094. However, further studies are needed to identify the actual

biochemical contribution of EptAs in *P. aeruginosa* species.

## CRedit authorship contribution statement

**Cindy Magda Araújo dos Santos Freire:** Conceptualization, Methodology, Data curation, Writing – original draft. **Alessandro Taunay-Rodrigues:** Visualization, Investigation. **Michelangelo Bauwelz Gonzatti:** Conceptualization, Methodology, Data curation, Writing – original draft. **Fátima Morgana Pio Fonseca:** Conceptualization, Methodology, Data curation, Writing – original draft. **José Ednéio da Cruz Freire:** Supervision, Validation, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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