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

Cindy Magda Araújo dos Santos Freire, Alessandro Taunay-Rodrigues, Michelangelo Bauwelz Gonziatti, Fátima Morgana Pio Fonseca, José Ednésio da Cruz Freire

**A R T I C L E   I N F O**

**Keywords:**
- Polymyxin
- Computational biology
- Bacterial resistance
- Gram-negative bacteria

**A B S T R A C T**

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) protein from *Escherichia coli* was used for comparison. Similar sequences to the protein MCR; 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 DUF1705 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: *colistinus Kayamae* (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 (Zavaski 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$_3$-T6SS, and cations replacement, minimizing the Mg$^{2+}$ concentration and increasing the Ca$^{2+}$ 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$_3$-T6SS in *P. aeruginosa* has three effector proteins, called Tse$_{1-3}$ (type VI secretion exporters 1–3), where Tse$_2$ and Tse$_3$ can cleave peptidoglycan associated with the bacterial envelope (Hood et al., 2010), and this function of H$_3$-T6SS is directly linked to antibiotic

---

* Corresponding author.

E-mail addresses: cindy.lab@hotmail.com (C.M.A.S. Freire), taunay.ale@gmail.com (A. Taunay-Rodrigues), gonzatti@unifesp.br (M.B. Gonzatti), fatimamorganapf@gmail.com (F.M.P. Fonseca), jednesio@gmail.com (J.E.C. Freire).

https://doi.org/10.1016/j.crmicr.2021.100042

Received 26 January 2021; Received in revised form 30 April 2021; Accepted 6 June 2021

Available online 22 June 2021

2666-5174/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license
resistance in biofilms (Zhang et al., 2011).

Furthermore, absence of 2-hydroxy-lauurate and presence of 4-aminooarabinose 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 (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 it is known as EptAs, whereas in the bacterium Escherichia coli it is known as MCR1, and Klebsiella pneumonia it is known as MCR-9. 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; Bomfilm 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 characterisation

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 Deleage, 1995). For protein domain prediction all .fasta extension sequences were submitted to Conserved Domains Database (CDD) and Resources - NCBI - NIH server (Li, 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 replicates 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 (Habib 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; Guru, 2013).

On the other hand, in microorganisms as Acinetobacter baumannii, E. coli and Klebsiella pneumonia 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. citrocellolis, 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 DUF705 superfamily, similar to proteins encoded by E. coli (Stoesser et al., 2016), Salmonella enterica (Douvith, 2016), K. pneumonia (Di Pilato et al., 2016), Serratia plymuthica strain AS13 e Neisseria meningitides (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

| Pseudomonas aeruginosa strains |
|--------------------------------|
| P. aeruginosa (taxid:287) |
| P. aeruginosa group (taxid:136841) |
| P. aeruginosa PA01 (taxid:209864) |
| P. aeruginosa PA103 (taxid:1099714) |
| P. aeruginosa DSM 50071 (taxid:1123015) |
| P. aeruginosa E2 (taxid:1163395) |
| P. aeruginosa str. PA17 (taxid:1333546) |
| P. aeruginosa PA1602 (taxid:1407059) |
| P. aeruginosa str. Stonet1 (taxid:1125697) |
| P. aeruginosa O3 (taxid:1402581) |
| P. aeruginosa BW25662 (taxid:1402528) |
| P. aeruginosa PA01 (taxid:1402553) |
| P. aeruginosa PA103 (taxid:381754) |
| P. aeruginosa BW25662 (taxid:1402545) |
| P. aeruginosa ATCC25234 (taxid:1163393) |
| P. aeruginosa VRPA15 (taxid:1431713) |
| P. aeruginosa UCBPP-PA14 (taxid:208963) |
| P. aeruginosa BW25662 (taxid:1402529) |

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/).
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 EptA enzymes showed that it is larger than other proteins from E. coli MCR, K. pneumoniae MCR-9, and S. enterica MCR-6 (GenBank ids: ASK04346.1, ARA472436.1 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 (Olatan 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 EptA isoforms 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 MCR1 of E. coli, MCR-9 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 MCR1 (60.12 kDa), K. pneumoniae MCR-9 (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 EptA proteins belong to the DUF7970 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. pneumonia (Di Pilato et al., 2016), S. plymuthica AS13 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 EptA 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 (MW), Theoretical (pl), Instability index (II), Aliphatic index (AI), and Grand average of hydropathicity (GRAVY) were obtained after access to the ProtParam tool (https://web.expasy.org/protparam/). All data revised and checked on April 20th, 2021.
isolectric 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* MCR1, and *K. pneumoniae* MCR1, 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 isolectric 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 (Guru-prasad et al., 1996). The instability index provides an inverse estimate of the metabolic stability of a protein (https://web.expasy.org/protparam/). 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* MCR1 and *S. enterica* EptA protein (Doumith, 2016; Di Pilato et al., 2016), 21.78 for *K. pneumoniae* MCR1 (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* MCR1, *K. pneumoniae* MCR1, *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* MCR1 and *S. enterica* EptA, 93.03 for *S. plymuthica* EptA, 93.36 for *K. pneumoniae* MCR1, 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 hydrophathy 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* EptA profiles 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* genome, while the other group of these enzymes may 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* MCR1, 0.067 for *S. enterica* EptA and 0.069 for *K. pneumoniae* MCR1, all with hydrophilic 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 PEEl 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 α-helices (44.15 - 47.68%), followed by loops (28.76 - 30.31%) and β-sheets (22.58 - 25.72%); (II) formed by 11 isoforms (35.49%) with predominance of loops (39.35 - 41.51%), followed by α-helices (30.75 - 34.18%) and β-sheets (26.12 - 28.61%). When comparing the data obtained for EptAs from *P. aeruginosa* with MCR1, in *E. coli*, MCR1, and 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 α-helices are predominant, followed by loops and β-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 thermos stability 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 DUPF705 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, MCR1 of *E. coli*, MCR1, and 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 dendogram 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 MCR1 proteins of *E. coli*, MCR1, 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 enzymes in the mechanism of the resistance to polymixines by members from *Enterobacteriaceae* family (Vilkaido, 2003; Alves and Behar, 2013; Andino and Hannig, 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
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 septicemia (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 EptA 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 MCR1 of *E. coli*, MCR1 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 DUF1705 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 Ednésio 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.

**References**

Al-Saleh, E., Akbar, A., 2015. Occurrence of *Pseudomonas aeruginosa* in Kuwait soil. *Chemosphere* 120, 100–107. https://doi.org/10.1016/j.chemosphere.2014.06.031.
Alves, A.P., Behar, P.R.P., 2013. Infecções hospitalares por enterobacterias produtoras de KPC em um hospital terciário do sul do Brasil. Rev. da AMRIGS 57 (3), 213–218.
Andino, A., Hanning, I., 2015. *Salmonella enterica*: Survival, colonization, and virulence differences among serovars. *Sci. World J.* 2015, 1–16. https://doi.org/10.1155/2015/S20179 no. ID 520179.
Barros, L.L.dos S., Maia, C.do S.F., Monteiro, M.C., 2016. Fatores de risco associados ao agravamento de sepse em pacientes em Unidade de Terapia Intensiva. *Cad. Saúde Coletiva* 24 (4), 388–396. https://doi.org/10.1590/1413-812320160040991.
Basso, M.E., Pulcinelli, R.S.R., do C. Aquino, A.R., Santos, K.F., 2016. Prevalência de infecções bacterianas em pacientes internados em uma unidade de terapia intensiva (UTI). *Rev. Bras. Anál. Clin.* 48 (4), 383–388. https://doi.org/10.21877/2448-3877.201600307.
Herrera, C.M., Hankins, J.V., Trent, M.S. 2010. Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. Mol. Microbiol. 76 (6), 1444-1450. doi.org/10.1111/j.1365-2958.2009.06544.x.

Habbu, P., Warad, V., Shastri, R., Madagundi, S., Kulkarni, V.H., 2016. Antimicrobial resistance of Pseudomonas aeruginosa PA01 grown at both body and elevated temperatures. Febs J. 4 (2223), 1-19. https://doi.org/10.1111/febs.12234.

Chang, K.C., et al., 2012. Clonal spread of multidrug-resistant Acinetobacter baumannii in eastern Taiwan. J. Microbiol. Immunol. Infect. 45, 37-42. https://doi.org/10.1016/j.jmii.2011.09.014.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1647-1649. https://doi.org/10.1093/molbev/msy098.

Khan, K., Al-Tabbakh, M., Al-Mahaidi, M., 2013. Polymyxin resistance of Pseudomonas aeruginosa PAO1 grown at both body and elevated temperatures. Front. Microbiol. 4 (2), 639-649. doi.org/10.3389/fmicb.2013.00422.

Olaitan, A.O., Morand, S., Rolain, J.M., 2014. Mechanisms of polymyxin resistance: A focus on the different stages of outer membrane biogenesis. Microb. Pathog. 66, 256-264. https://doi.org/10.1016/j.micpath.2014.09.007.

Lopes, H.V., 2009. O tratamento das infecções graves por Pseudomonas aeruginosa. Rev. Pediatria Infect. 11 (3), 74-81. doi.org/10.11606/s1518-37472009000300003.

Li, J., Nation, R.L., Milne, R.W., Turnidge, J.D., Coulthard, K., 2005. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. Int. J. Antimicrob. Agents. 25 (3), 11-25. https://doi.j. jantimicag.com/2004-10010.

Liu, Y.Y., et al., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular epidemiological study. Lancet Infect. Dis. 16 (2), 161-168. https://doi.org/10.1016/S1473-3099(15)00424-7.

Lopes, H.V., 2000. O tratamento das infecções graves por Pseudomonas aeruginosa. Rev. Pediatria Infect. 11 (3), 74-81. doi.org/10.11606/s1518-37472009000300003.
Zhang, L., Hinz, A.J., Nadeau, J.P., Mah, T.F., 2011. *Pseudomonas aeruginosa tssC1* links type VI secretion and biofilm-specific antibiotic resistance. J. Bacteriol. 193 (19), 5510–5513. https://doi.org/10.1128/JB.00268-11.

Zhang, Q., Smith, J.C., Zhu, Q., Guo, Z., MacDonald, N.E., 2012. A five-year review of *Pseudomonas aeruginosa* bacteremia in children hospitalized at a single center in southern China. Int. J. Infect. Dis. 16 (8), e628–e632. https://doi.org/10.1016/j.ijid.2012.03.014.

Zheng, B., et al., 2018. Discovery and characterisation of an *Escherichia coli* ST206 strain producing NDM-5 and MCR-1 from a patient with acute diarrhoea in China. Int. J. Antimicrob. Agents 51 (2), 273–275. https://doi.org/10.1016/j.ijantimicag.2017.09.005.