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Escuela de Ciencias Biológicas e Ingeniería

Computational analysis of electrogenesis gene sequences in microorganisms

Trabajo de integración curricular presentado como requisito para la obtención de Título de Ingeniero Biomédico

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Dedication

This work is dedicated to my parents Rosa and Helio. They are the fundamental pillar in my life. They always encourage me to continue and conclude my academic studies; besides that, they always knew how to support me in the different adversities that arose during my university life. Thanks to my parents for the motivation that they have given me to remain motivated and persevering on the student journey. I will always be grateful to you.

At the same time, I want to dedicate this work to my tutors Marco, Mariel, and Carlos for being great mentors who with their excellent scientific background, were able to transmit knowledge related to the beautiful field of biological sciences and guide me throughout the entire which has been of great inspiration in my professional life.

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RESUMEN

El estudio de los microorganismos exoelectrógenos ha tenido un gran interés en las últimas décadas ya que su uso dentro de los sistemas bio-electroquímicos en especial en las celdas de combustible microbianas (MFC) ha tenido un gran impacto como una fuente alternativa para generación de electricidad en lugar de las formas de producción convencionales. Estos microorganismos presentan una serie de proteínas específicas que les permiten transferir los electrones hacia aceptores externos en las celdas de combustibles microbianas lo que les permitirá aprovecharlos para la producción de electricidad. Un análisis bioinformático de las tres proteínas principales involucradas en el proceso de transferencia de electrones como son MtrA, MtrC y PilA fue realizado con el propósito de encontrar similitudes estructurales y funcionales entre diferentes especies de microorganismos exoelectrógenos con el fin de apoyar estudios futuros enfocados en el desarrollo de cepas mucho más competentes que permitan el desarrollo eficiente de las celdas de combustibles microbianas a escalas industriales. Los resultados sugieren similitud estructural entre proteínas homólogas tales como MtrA y MtrC, e incluso con PilA sugiriendo la conservación entre diferentes filos como un mecanismo de sobrevivencia entre las bacterias.

Palabras clave: Celdas de combustibles microbiana, análisis bioinformático, secuencias conservadas, alineamiento múltiple de secuencias.

ABSTRACT

The study of exoelectrogenic microorganisms has had a great interest in recent decades since their use within bio-electrochemical systems, especially in microbial fuel cells (MFC). It has greatly impacted as an alternative source for electricity generation instead of conventional forms of production. These microorganisms present a series of specific proteins that allow them to transfer electrons to external acceptors in microbial fuel cells, which will allow them to take advantage of the production of electricity. Bioinformatics analysis of the three main proteins involved in the electron transfer process such as MtrA, MtrC, and PilA was carried out in order to find structural and functional similarities within different species of exoelectrogenic microorganisms in order to support future studies focused on the development of much more competent strains that allow the efficient development of microbial fuel cells on an industrial scale. The results show structural similarities in analogous proteins such as MtrA and MtrC; and even PilA suggesting a conservation among different phyla as a survival mechanism in different bacteria.

Keywords: Microbial fuel cells, bioinformatics analysis, conserved sequences, multiple sequence alignment

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Introduction

Exoelectrogen microorganisms

These microorganisms have evolved the capacity to transport electrons from their interior to external electron acceptors outside the cell by different metabolic mechanisms (1). This process is called extracellular electron transfer (EET). Since the first observations of the ability of several microorganisms as bacteria, archaea, and fungi to release electrons extracellularly by Michael Cressé Potter in 1911 (Figure 1), a great interest to take advantage of this process has emerged to create bio electrochemical systems (BES) to produce alternative ways for electricity generation(2).

Figure 1. Michael Cressé Potter (1859-1948) Botanist who introduced the idea of using microbes to produce electricity. (Figure from reference (3)).

These exoelectrogenic microorganisms are found in several environments such as: lagoons, oceans, groundwater, sediments, soils, swamps, rocks and even inside several superior animals, where they play an essential role in the nutrient cycles (4). EET occurs by redox activities during the metabolism of different substrates. For example, organic materials like carbohydrates, some proteins, heavy metals, sewage, and a great variety of compounds can serve as electron donors (5). In the laboratories, the principal mechanism to study these microorganisms is through the culture using metallic electron receptors such as iron and manganese oxides (6).

Several microorganisms have been tested to assess the capacity to release electrons to external acceptors. Among them, members of the genera *Geobacter* and *Shewanella* have been the most studied. However, other species have shown an EET ability, such as *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Escherichia coli* , *Klebsiella pneumoniae, Enterobacter cloacae, Tolumonas osonensis, Proteus vulgaris, Comamonas denitrificans, Alcaligenes faecalis, Erwinia dissolvens, Ochrobactrum anthropic* among others (7) (8).

Over time, several exoelectrogen microorganisms (EM) have attracted the attention of the scientific community for their applications. Different Bioelectrochemical systems (BES) have been developed to get their benefits like bioenergy production (9). These devices (Figure 2) can get the electrons delivered by EM through electrodes which will transport those to an external circuit that will use the generated voltage to guarantee power generation output, likewise electricity production (10). The ways by which electrons are transferred depend on the biofilm formation on the anode surface by protein complexes as cytochromes, nanowires and mediators.

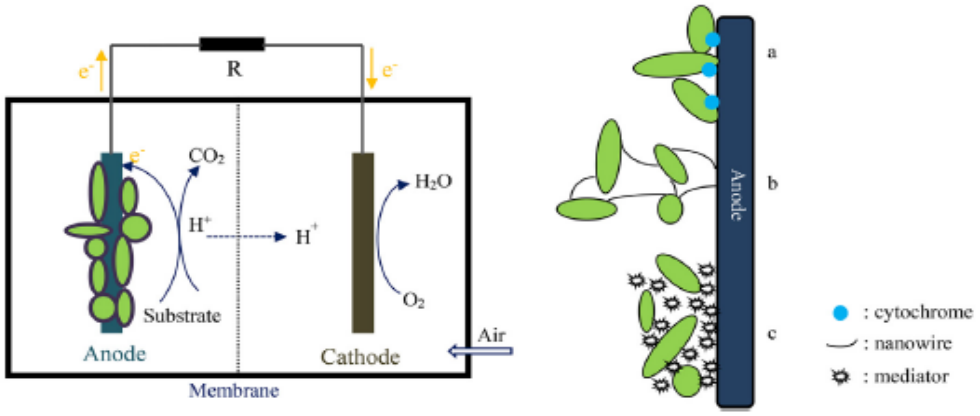

Figure 2. Bio electrochemical system. It is used to take advantage of EM metabolism for bioelectricity generation. The anode chamber storages the substrate that EM will oxidize and transport the electrons through their metabolism and release them to the anode that will conduct the electrons through an external circuit to the cathode to produce water to the reaction of protons, electrons, and oxygen located in the cathode chamber. (Figure from reference (4)).-

Conditions for EET

Chemical and physical factors may affect the capacity to transfer electrons to their acceptors. Among them, pH, oxygen concentration, type of substrate, and temperature define the optimal conditions to assure the bacterial metabolism and electron transference.

pH.

Microorganisms can grow up at different pH conditions such as acidic or alkaline. Species of the genus *Geobacter spp.* preferably live in environments with pH values between 6.2 and 7.4 in anaerobic conditions (11). On the other hand, optimal conditions of pH for *Shewanella spp.* oscillates between 5.8 to 7 (12). These data suggest the broad adaptive conditions of these microorganisms to different environments which could support its application at power generation processes (13).

Oxygen concentration

Exoelectrogen microorganisms may be aerobic or anaerobic depending on the final electron acceptor during the electron transference chain (14). The exoelectrogenic microorganisms are preferably, anaerobes or facultative using alternative acceptors in the respiratory process such as the oxidized forms of iron and manganese (5) (15).

Environmental sources of exoelectrogenic microorganisms

EM tends to form mats and biofilms that support the survival of the community in some environments and have complex behavior that is still a vast source of research (16). Therefore, microorganisms establish communities in all environments. Some of them are accessible to human beings, and others are too extreme to favor the development of the most eukaryotes (17).

Even at the most extreme conditions, exoelectrogen microbes can get their energy source to be used in their metabolic pathways. For instance, domestic sewage (17), waste from paper processing (18),brewery wastes (19), manure (20) (16), polluted effluents (21) (22), among others.

Temperature

Since most proteins and enzymes involved in metabolism require an optimal environment to perform their functions (23), another critical factor in the EET process is temperature. High temperatures may trigger denaturalization of proteins, loss of efficiency, and finally death of the microorganisms. Several microorganisms have adapted to different temperature conditions,

and that is the advantage of working with them in artificial environments in BES for power production(24).

Laboratory conditions tend to replicate the optimal growth temperatures of EM. Species of *Firmicutes spp*., *Proteobacteria spp*. and *Termicola spp*. can survive at high temperatures avoiding denaturalization of their proteins. Consequently, these species have been considered as biotechnological tools during EET processes (13) (25).

Extracellular Electron Transfer (EET)

Some microorganisms can transfer electrons from the cytoplasm to extracellular environments, due to their proteins which perform redox reactions using organic substrates as electron donors and metals as acceptors (Figure 3) (26). Microorganisms use several pathways to allow this process, such as direct electron transfer, allowing the electron transference to another microorganism or to external acceptors (27).

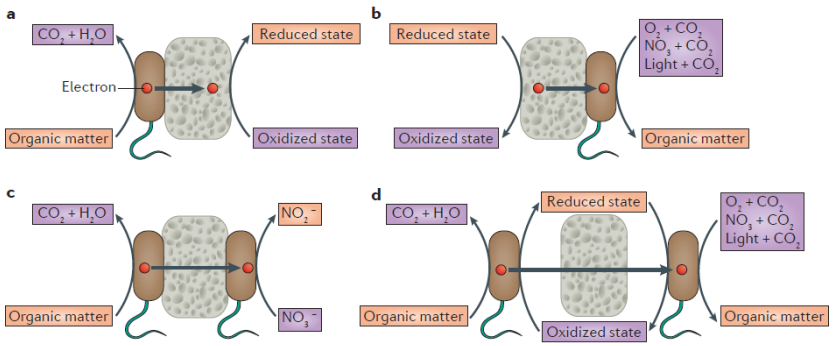

Figure 3. EET between the microorganism and minerals. (a) Transference from microbe to the mineral. (b) Transference from mineral to the microbe. (c) Interspecies electron transfer and (d) Storage of electrons in minerals (Figure from reference (28)).

Transmembrane electrons transfer (TET) describes the entire process by which the electrons enter the microorganism after being transported during the metabolic pathways and finally released to the exterior (27). Remarkably, each EM has its metabolic route to release electrons outside the cell and their respective genes regulating the whole process (29).

Extracellular electron transfer is possible thanks to several molecules that act both inside and outside the cell. Generally, the transference could be possible directly and indirectly by some pathways. The principals are through the outer membrane cytochromes, electron shuttles and the pili (30).

Outer membrane Cytochromes(*c*-Cyst)

The *c*-Cyst are proteins associated principally with the inner and outer membrane. They are the critical molecular component of the electron transport chain. These molecules belong to the cytochrome c family (6). Although the *c*-Cyst (Figure 4) are found in the genera *Geobacter spp.* and *Shewanella spp*, the ability to release electrons outside the cell is different for each one.

Thus, EET in *Geobacter spp.* is carried out by multi-haem c-Cysts and multi-copper proteins such as ImcH and CbcL located in the cytoplasmic membrane. The PpcA, PpcD, Omas and Omcs in the periplasmic space and the OmbB and OmbC of the outer membrane are responsible for the electron flow through the bacterial membranes (30). Instead of that, *Shewanella spp.* presents another structure of six muli-haem c-Cysts that regulate the EET by other molecules as CymA in the cytoplasmic membrane, the Fcc3 small tetrahaem cytochrome (SRC) for the periplasm, and the MtrCBA in the outer membrane (30).

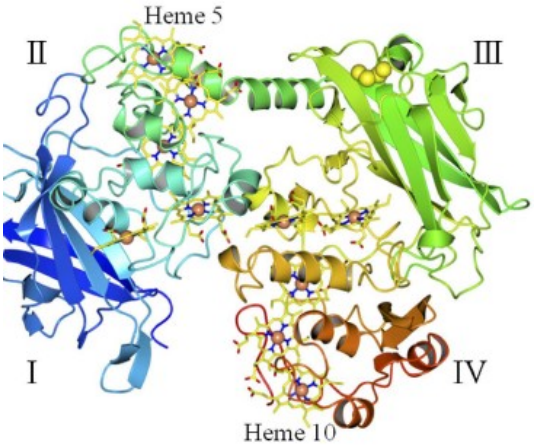

Figure 4. Structure of MtrC. One of the cytochromes that are found in the outer membrane of *Shewanella spp.* (Figure from reference (31))

Electron Shuttles

These intracellular molecules are located both inside the cytoplasm and the periplasm. They act as carriers of electrons outside of the plasmatic membrane. These molecules play the role of co-factors that bind to cytochromes and form a complex that allows electron transfer to the electron acceptors (10).

Among these electron shuttles are: flavins, flavocytochrome, phenazines, Flavin mononucleotide and riboflavin (32) These small molecules are very effective due to their fast capacity to turnover from oxidized forms to reduced forms (33).

Nanowires

Conductive proteins, also called nanowires or electrical filaments, are surface proteins located on the plasmatic membranes of some EM allowing a direct electron transference. These nanowires are in direct contact with electron acceptors and lead to new concepts for long-range electron transfer (34). These natural appendages play an essential role. For example, pili for *Geobacter spp.* are able to use Fe (III) as electron acceptor in soils and sediments (35).

Several studies have shown that conductive pili are formed by several monomers of pilin encoded by the gene *pilA* that perfectly assembles that structure (36) (37) (38).

Genes involved in exoelectrogenic activity

Exoelectrogen Microorganisms have several metabolic routes that allow EET. Moreover, each organism has different ways by which they transfer electrons extracellularly, which depends on physiology (39)(40).

Within the genomes of microorganisms related to EET, it has been identified around 117 genes associated with c-type cytochromes. From these ones, at least 16 play a crucial role for electron transfer (6). Additionally, other variables such as the potential of electron acceptors, salinity, temperature, the biofilm interaction, and even the material of the electrode are linked with the regulation of different functional genes associated with the electron flow.

Electron shuttles, cytochromes, and pili are the most critical mediators until now that make possible EET. Thus, several genes associated with them have been found in exoelectrogenic microorganisms. For example, deletion of the gene *pilA*, which forms the structural pilin protein, eliminates high current production capacity, suggesting that pili are important in electron transfer (41). In other cases, as in *Shewanella spp*. The MtrCAB protein complex is the primary route for electron transfer in which genes *mtrA, mtrB*, and *mtrC* encode the proteins to form the membrane complex that carry electrons from the periplasm to outside, and their elimination shows a reduction in the current generation (42)(35)(43)(44).

This work principally focused on three proteins involved directly in the extracellular electron transfer. Two of them are MtrA and MtrC, which form the Mtr protein complex which is associated with electrons transported outside the cells; MtrA receives electrons from the menaquinone pool with the aid of cytochromes. Instead, MtrC interacts with external acceptors,

which is essential for the molecular correlation between them for the final electron transference (45).PilA or pilin is the structural protein of the pili. This structure has a different way of transporting electrons due to its capacity to transfer electrons to the extracellular acceptor (6) (38) (41) (42).

Bio electrochemical systems (BES)

The principal function of these devices is the use of different types of microorganisms to transform the chemical energy stored in substrates into electrical energy (5). Through the potential capacity of EM, these have been used in BES to solve several acute problems such as contamination of soils and water and electricity production.

This technology and its development use multidisciplinary knowledge for its creation and integrates areas as material sciences, engineering, electrochemistry, mathematics, nanotechnology, microbiology, and other related fields (3). Moreover, these technologies are so flexible that they allow different uses depending on the purpose. The principle of these devices is to take advantage of EET for the current generation. For this case, the type of BES is called microbial fuel cells (MFC) (46). Nevertheless, these technologies could be used for similar purposes; BES for H2 production are called microbial electrolysis cells (MEC). Others in which the configuration use electrons in the cathode chamber to synthesize organic compounds are called microbial electrosynthesis (MES), or others to remediate contaminated water are called Microbial Desalination cells (MDC), as is shown in the figure 5 (47)(19).

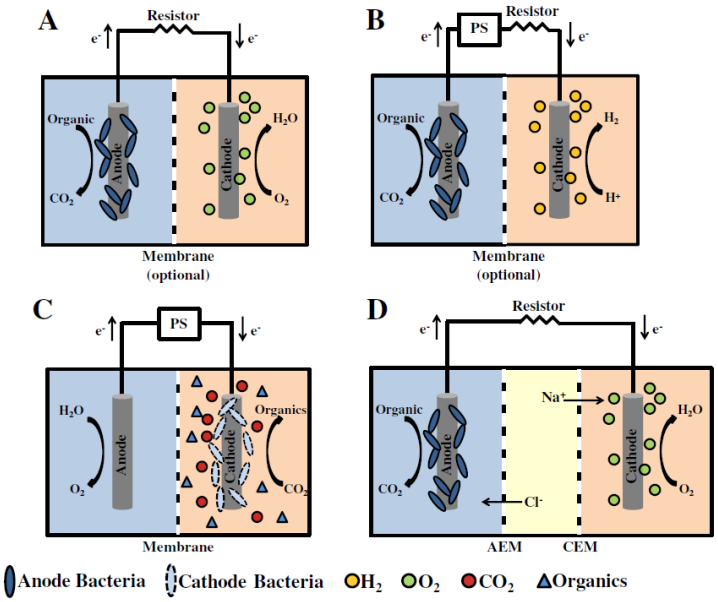

Figure 5. Basic structure of different BES. a) Microbial Fuel Cells. b) Microbial Electrolysis Cells. c) Microbial Electrosynthesis and d) Microbial Desalination Cells (Figure from reference (5)).

Justification

The vast majority of the energy sources are based on fuel derived fossils. Principal troubles related to its consumption are associated with the environmental pollution and the high production costs. One alternative to this situation is the green electricity production. The exoelectrogenic microorganisms are a potential alternative to the use of conventional energy and electricity sources due to their unique property to transfer electrons extracellularly to electrodes in several bio electrochemical systems.

With these antecedents, bioinformatics tools can provide enough information about the common conserved residues in the MtrA, MtrC and PilA proteins which are involved in the exoelectrogenic processes. These results could serve as the basis for future studies focused on the development of efficient strains as a tool to research and industrial activities.

Objectives

General Objective

Perform a computational analysis of electrogenesis genetic sequences of microorganisms capable of extracellular electron transfer (EET) to understand the relationship of proteins involved in electron transference in different Exoelectrogens Microorganisms (EM).

Specific Objectives

1. Perform computational analysis of homologous sequences to find different bacterial genera capable of extracellular electron transfer and specific proteins related to that process.

2. - Analyze the protein sequences involved in the process of electron transfer to indicate functional correlation between these proteins inside Exoelectrogenic Microorganisms.

Methodology

1. - Sequence Selection

The proteins used in this project were selected based on their capability to form protein complexes that can release electrons outside the cells and participate in the exoelectrogenic process for bioelectricity production (51)(16)(9).

Consequently, the development of this work was focused on three proteins MtrA, MtrC and PilA (4)(48)(10) related to the process of Extracellular Electron Transfer (EET)(49)(40)(50) (51)(16)(9).

The selected proteins were cataloged as "*reference sequences*" and are shown in Table 1.

Table 1. Reference sequences used in this study. It is shown the bacterium name, the proteins and their accession number.

Bacterium	Protein	Accession number
Geobacter bremensis	MtrA	BCG46335.1
Shewanella putrefaciens (strain CN-32)	MtrC	NC_009438.1
Geobacter bremensis	PilA	BCG47856.1

2. -Finding protein homologous sequences

The reference sequences were used to find homologous protein sequences to establish areas of similarity which could indicate functional or evolutionary relationships among proteins in different bacteria. The homologous sequences were obtained from the web server JackHmmer (version 2.41.1) (52), this server performs fast and sensitive homology search (52). The sequences were retrieved from JackHmmer using UniProtKB as the source database (53).

Once it was performed, with the purpose to ensure that all sequences were retrieved, BLAST (Basic Local Alignment Search Tool) (54) was used to obtain homologous sequences to find those that have the most similarity with the reference sequences. The protein sequences were obtained using the UniprotKB database under the protein-protein blast algorithm.

3. - Sequence Cleaning

Once the total number of sequences for each protein were retrieved, the protein sequences were concatenated using CAT command through linux terminal, this was done to create a single file with the information from Jackhmmer and Blast. Nonetheless, having used different software that compares protein sequences to a repository of sequence databases it was expected to find repeated information. In order to eliminate repeated sequences, the command **seqkit rmdup-s** through linux terminal was used.

Finally, the program SeqScrub (55) allowed to remove outdated sequences (55). SeqScrub manages curation options that allow it to remove obsolete and un-mappable sequences.

4. – Sequence alignment

4.1 Multiple Sequence Alignment

Multiple Sequence Alignment (MSA) was performed to infer the homology among the protein sequences (56) . To carry out the MSA, three different software were used: MEGAX (Version 10.2.2) (54), Seaview (Version 5.0.4) (57), and Jalview (Version: 2.11.1.3) (58) all of them use MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm (59) (56). MEGAX and Seaview were used to make the alignment of the sequences. These programs align more than one thousand sequences and export the alignments to present MSA images (57). Jalview allows the visualization of the consensus sequences and the identity percentage that helps to differentiate the MSA results (58).

5. – Sequences logo representation

To obtain the graphical representations of amino acid multiple sequence alignment, it was necessary to use Weblogo 3 (60) which provides a more accurate and easy description of the sequence conservation and gives a quick view of the amino acids characteristics in the alignment. For this study, different parameters were used to understand the features of conserved regions. Among them were considered: the hydrophobicity of the residues; their polarity and their electrical charge. The sequence logos were retrieved from Weblogo 3 under this condition: the units used for the y-axis were changed to show the residue appearance probability in the alignment.

Results and Discussion

Screening of exoelectrogenic proteins

The reference sequences of MtrA, MtrC, and PilA were used to retrieve homologous protein sequences from the JackHmmer web server (version 2.41.1) (52) and from Blast P (61) as shown in Table 2.

Table 2. Sequence screening from JackHmmer and Blast P. Resumes the total number of sequences retrieved in each case.

Protein	Sequences from JackHmmer	Sequences from Blast P
MtrA	2466	45
MtrC	800	250
PilA	417	250

SeqScrub (55) allowed the cleaning of repetitive and un-mappable sequences between those that were retrieved from JackHmmer and Blast P.

The total number of sequences before and after cleaning are shown in Table 3.

Table 3. Sequence cleaning with SeqScrub. It is shown the total number of concatenated sequences and the total amount of cleaned sequences.

Protein	Concatenated sequences	Cleaned sequences
MtrA	2511	2198
MtrC	1050	661
PilA	667	432

After the cleaning procedure, 500 sequences from MtrA, 500 sequences from MtrC and 432 sequences from PilA were chosen to continue the analysis. This process was made to avoid data

bias when obtaining the logos since most of the amino acids within the alignments would be predominated by MtrA and MtrC proteins over PilA. Finally, a total amount of 1429 protein sequences were aligned.

Alignment of the sequences and bioinformatics analysis.

There has been a tremendous amount of evidence that the Mtr proteins complex is involved in the extracellular electron transfer, but the mechanism by which this process is carried out remains unknown (62) (63) (64). This complex is formed by the two cytochromes: MtrA in the periplasmic space of the cell and MtrC that is faced to the extracellular space. Furthermore, pili have another protein structure and act as an important factor during the electron transference because it allows direct electron transport to extracellular acceptors (38) (65). The bioinformatics analysis reported in this study revealed the different roles of the conserved residues related to Mtr proteins inside exoelectrogens microorganisms.

Across all 1429 sequences analyzed, three high conserved regions were found: one from the residue 140 to residue 150; the second one from the residue 2141 to 2147, and the last one from the residue 3931 to residue 3931. This could suggest us possible functions of these residues inside the proteins:

The first identified region in the alignment of the three proteins is located in the proximal sense to the amino terminal region, concretely from the residue 140 to the residue 150, as shown in Figure 6A. This region had a noticeable amount of residues that share essential properties as hydrophobicity and neutrality (Figure 6B). This conservation grade can be associated with structural characteristics of the proteins, especially to maintain the stability during the protein folding (31).

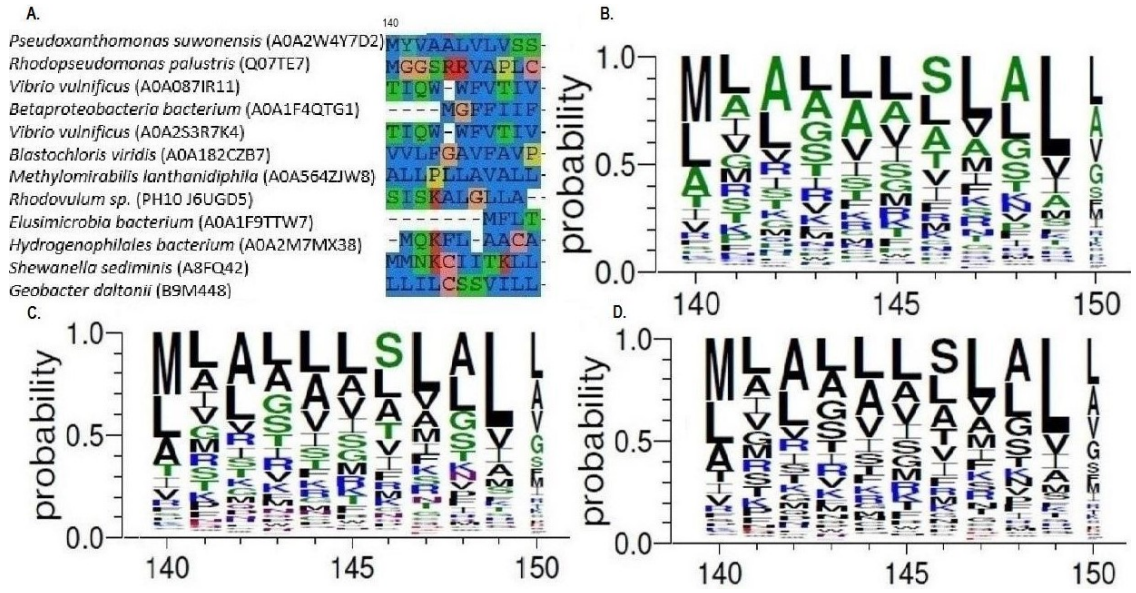

Figure 6. Multiple sequence alignment of MtrA, MtrC, and PilA proteins between the 140 to 150 residues. A) The figure shows a fragment of the entire sequence alignment that best expresses the sequence logo. The identity of the amino acids is given by different colors, which indicate the shared properties between them as blue, which means hydrophobicity (M, V, A, L, F), and green is polar (S, T, Q).**B)** Sequences logo for hydrophobic properties, indicating if the amino acid could be hydrophilic, neutral, or hydrophobic. The most conserved amino acids are methionine, valine, and leucine which are hydrophobic (Black). Alanine, glycine, and serine are neutral (Green). **C)** Sequence logo for chemical properties, indicating if the amino acid could be polar, neutral, basic, acidic, and hydrophobic. The most conserved amino acids are methionine, leucine, alanine, and valine which are hydrophobic (Black). Glycine and serine are polar (Green). **D)** Sequence logo for charge properties, indicating if the amino acid could be positive or negative. The most conserved amino acids are methionine, leucine, alanine, glycine, serine, and valine which are non-charged (Black).

Thus, residues as leucine and methionine predominate in this region which allow the folding along the chain of the amino acids. These residues do not have an asymmetric distribution of the charge that supports the aggregation of these molecules (66) (Figure 6D). Other essential residues that showed hydrophobic and aliphatic properties are valine, alanine, methionine, and leucine contributing to the tertiary structure of the protein through the formation of helixes (67) (Figure 6C).

In this region, there is evidence that the majority of conserved residues are methionine, leucine, and alanine. These amino acids, instead of the binding motif CX_nCH, which represents the residues cysteine followed by any amino acid with n>2 and after cysteine and histidine, are considered another binding motif that could make only a single thioether linkage with the heme group (68); in this case, the methionine will replace the function of histidine to coordinate the iron in the heme group (69) (70) (71). That reaction is important for the catalytic redox processes to accept and release electrons inside the cytochromes (72).

Another region was found from the residue 2141 to the residue 2147, as shown in Figure 7A. According to figure 7B, the most conserved amino acid is cysteine. This fact is very important due to their hydrophobic and non-charged characteristics, which have a high tendency to form helix structures and have high turn propensity (70).

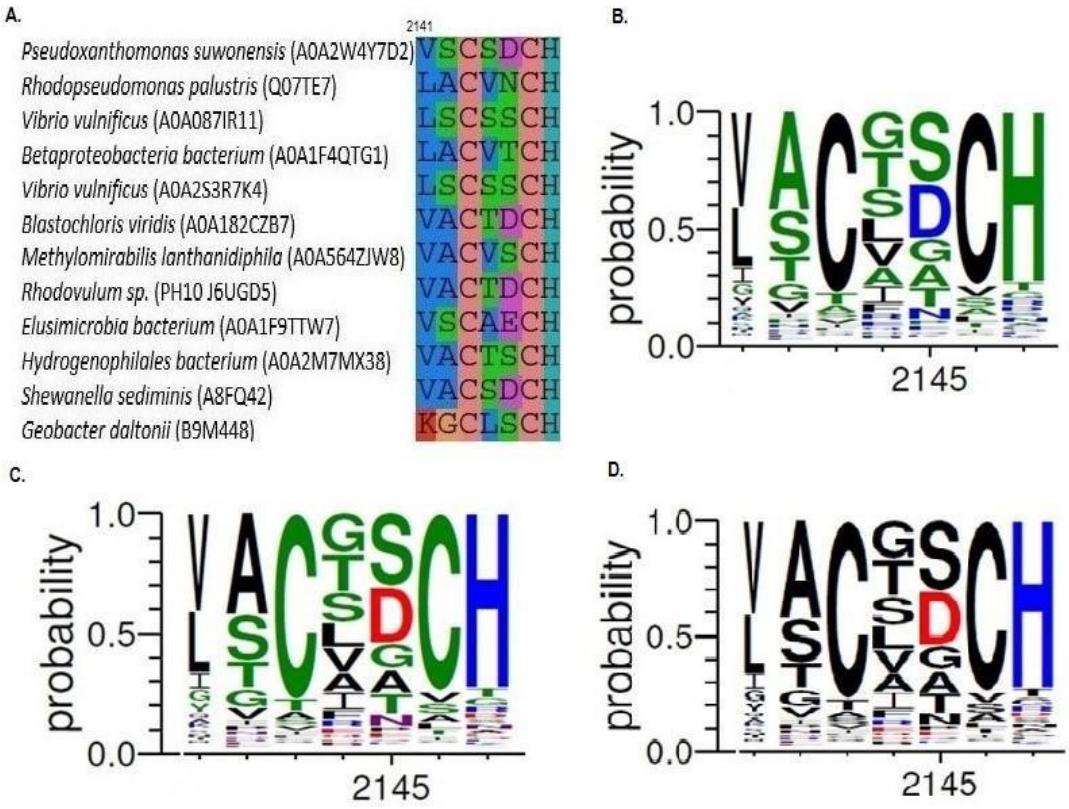

Figure 7. - Multiple sequence alignment of the MtrA, MtrC, and PilA proteins between the 2141 and 2147 residues. A) The figure shows only a fragment of entire sequences to express the similarity with the sequence logo. The colors indicate different residues characteristics as green

and blue that represents polarity; orange represents neutral amino acid, and magenta means hydrophilic. **B)** The sequence logo for the region 2141 to 2147 shows hydrophobicity characteristics, indicating hydrophilic, neutral, or hydrophobic properties. The most conserved amino acids cysteine, valine and leucine are hydrophobic (Black). Glycine, threonine, alanine and serine are neutral (Green). **C)** The sequence logo shows the essential amino acids for chemical properties, indicating if the amino acid could be polar neutral, basic, acidic, and hydrophobic. The most conserved amino acids are leucine, valine, and alanine which are hydrophobic (Black). Aspartic acid is acidic (Red). Histidine is basic (Blue), and cysteine, glycine, threonine and serine are polar (Green). **D)** The sequence logo for this region shows the charge properties, indicating if the amino acid could be positive, negative, or non-charged. The unique amino acids that show negative and positive charge properties are glutamic acid (Red) and histidine (Blue), respectively; and the others as valine, leucine, alanine, threonine, glycine and serine are non-charged(Black).

Other conserved residues are aspartic acid, that is hydrophilic, and alanine that is polar (Figure 7C). These amino acids have the properties and tendency to form helix structures but less threonine, serine and glycine. On the other hand, these amino acids have a low propensity to form any strand structures; instead of that, serine and glycine, due to its neutral and non-charged nature, as shown in figure 7D, have a high turn propensity that contributes to the stability of the structure domain (70).

Another conserved region was found proximal to the carboxyl-terminal between the residue 3931 and 3937 (Figure 8A). Due to the negative charge, acidic and hydrophilic properties of glutamic acid and neutral properties of Alanine (Figure 8B, 8C, 8D), they have a high propensity to form helix structure and low tendency to form strand structures(Figure 8B) (73). Instead of that, serine residue in this region contributes to high turn propensity, which helps folding stability as mentioned above (69).

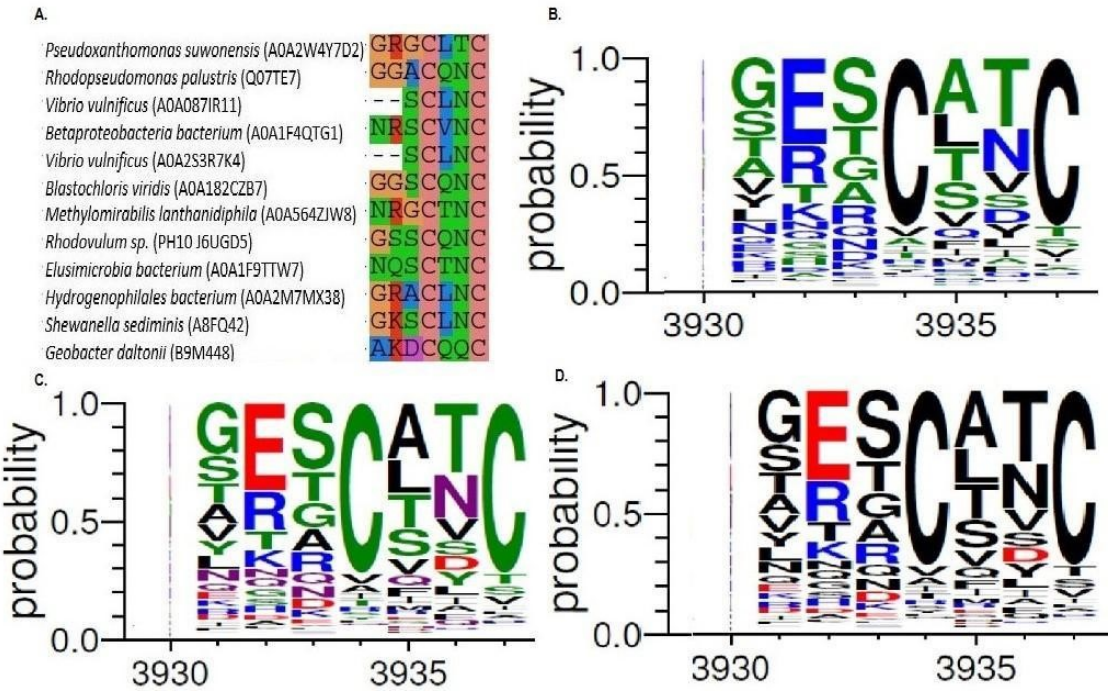

Figure 8. - Multiple sequence alignment of the region from 3931 to 3937. **A)** The figure shows only a fragment of all sequences in the alignment. The colors in the figure indicate the different properties of residues as orange means neutral; blue and green represent hydrophobicity, and magenta is a hydrophilic amino acid. **B)** This logo shows the hydrophobicity properties of residues. Cysteine is hydrophobic represented with black color; simultaneously, glutamic acid, arginine, and asparagine are hydrophilic, represented with blue color and finally, the neutral residues represented with green color are glycine, serine, threonine and alanine. **C)** This logo represents the chemical properties of the amino acids in that region. Several colors represent polar, neutral, basic, acidic, and even hydrophobic amino acids. The most representative residues are polar, shown with green color, cysteine, glycine, serine, and threonine. Black color represents the hydrophobic amino acids as alanine and leucine, while red and blue are acidic and basic. The purple color represents neutral amino acids as asparagine. **D)** The logo that represents the charge of each residue. The most representative amino acids in this region are non-charged as cysteine, serine, threonine, glycine, alanine, and asparagine, represented with black color. The other ones are a red and blue color representing negative and positive charge: glutamic acid and arginine, respectively.

In the region from 2141 to 2147 it was found a highly conserved cysteine and histidine residues, which are very important due to this region is a binding motif CX_nCH (74) (75) which means the

residues cysteine followed by any amino acid with n>2 and after a cysteine and histidine. This binding motif is very important due to it allows the attachment of heme functional group by two thioether bonds by cysteine instead of only one such as methionine and the other is that histidine will allow the coordination of iron in the heme structure.

The last region from 3931 to 3937 present highly conserved cysteine residues, but instead of the two regions this do not present histidine or methionine that could allow the coordination of iron in the heme complex, but the pattern suggest the same motif as CX_nC that could indicate the attachment of heme group through to thioether bonds but with another type of iron atom coordination (76)(31) (66).

It is essential to mention that the heme structure has an iron atom in the middle, and this is the fundamental actor due to this the atom will be reduced or oxidized, being in different forms as ferrous (Fe^{2+}) and ferric (Fe^{3+}) that will allow the electron transport (77).

As mentioned above, the genera *Geobacter* and *Shewanella* are the most studied microorganisms due to their capacity to transfer electrons outside the cell. After the analysis of the 1429 homologous sequences of the proteins MtrA, MtrC and PilA, we found 5 genera that shared a grade of conservation among their residues. These microorganisms correspond to the genera: *Acidobacter* spp., *Desulfuromonas* spp., *Ferrimonas* spp., *Nitrsopira* spp. and *Vibrio* spp.

Vibrio spp. like *Shewanella* spp., use the complex Mtr and CymA as the molecular mechanism to transfer electrons from the inner of the cell (78) (79) (80). Similarly, *Ferrimonas* spp also is able to transfer their electrons through the complex Mtr (83) (84). On the other hand, the genus *Desulfuromonas* has been described as an exoelectrogenic microorganism due to the capacity of their pili to transfer electrons outside the cell (82).

Although *Acidobacteria* spp. and *Nitrospira* spp. have been described as exoelectrogenic microorganisms (86, 87), there is not enough evidence of the molecular mechanisms responsible for this process. In a whole, these results suggest that future studies could be conducted with the purpose to elucidate the molecular mechanisms that regulate the exoelectrogenic process of these microorganisms.

It is remarkable to take into account that due to the alignment of sequences were between two proteins that form the Mtr complex MtrA and MtrC and the protein of pili structure, PilA, there are many conserved residues of cysteine, histidine, methionine, and alanine between these

three proteins of different species; this could suggest a possible common organization among organisms with the purpose of maintain the exoelectrogenic function in different phyla of bacteria (31) (63) (66) (71).

The analysis of these sequences could introduce a future study focused on the relevant amino acids motifs, and even study the relationship between the electron transference of Mtr proteins and pili based on the redox activity for electron transfer. Also, as previously described, the heme groups are principally coordinated with histidine residues instead of methionine and also due to redox potentials of the heme groups vary from 0 to 400 mV (69) (88) this could serve as a solid basis to create genetically modified strains that could have histidine residues over methionine to aid in the attachment of heme groups as potential bioengineered tools with improved capacity to the electron transference process.

Furthermore, the results show the most conserved region of the major of exoelectrogens proteins involved in extracellular electron transfer, and this could suggest how the constant evolution rate of the cytochromes and pili can be a powerful tool to identify when these microorganisms may diverge from a common ancestor due to these share motifs that were presented in the multiple sequence alignment (89).

Conclusions

Exoelectrogen microorganisms have been called the attention of the scientific community for their exoelectrogenic activity, which are used in electricity production through microbial fuel cells. Thanks to JackHmmer and Blast P web servers, homologous sequences from the reference sequences were obtained rapidly for the posterior bioinformatics analysis. In total, 1429 sequences from the proteins related to extracellular electron transfer MtrA, MtrC, and PilA were analyzed to find structural and functional relationships between them.

The multiple sequence alignment found three principal conserved regions between all species that share the mechanism to transport electrons extracellularly. These regions were: from 140 to 150 residue; from the residue 2141 to 2147 and the final region between the amino acid 3931 and 3937. Among them, the principal binding motifs related to the attachment and coordination of heme groups through cysteine, histidine, and methionine are the most conserved residues inside these regions in the majority of analyzed bacteria.

These findings demonstrated that pili and Mtr proteins probably share the same binding motifs and this could suggest these proteins uses heme structures for electron transferring outside the cells; having in mind that MtrA and MtrC are closely related but accomplish different functions in the inner and outer space of the cell and even pili structure is considered as a direct electrons transporter this could use the heme structure to transport electrons outside the cells.

The alignments suggest an understanding of the relationships of the most relevant proteins associated to the extracellular electron transference process. It has been established here a fundamental relation among MtrA, MtrC, and PilA proteins due to their shared properties in conserved regions.

Future work will be necessary to define and compare the different functional and structural domains between other correlated proteins in extracellular electron transfer to understand the detailed characteristics of its mechanisms.

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