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Identification of Key Proteins in Heme Uptake by *Pseudomonas aeruginosa* by *In Silico* Analysis: New Therapeutic Targets

Identificación de Proteínas Clave en la Captación de Hemo por *Pseudomonas aeruginosa* mediante Análisis *In Silico*: Nuevos Blancos Terapéuticos

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ABSTRACT

Pseudomonas aeruginosa (P. aeruginosa) is a Gram-negative bacterium of significant clinical relevance, causing severe nosocomial infections, particularly in immunocompromised patients. Its ability to acquire iron plays a crucial role in its pathogenicity. This study aimed to identify key membrane proteins involved in heme uptake as potential therapeutic targets. To achieve this, we performed an *in silico* analysis, assessing 47 potential membrane proteins using *ExPASy BLAST*. This analysis identified seven candidate membrane proteins that contain the conserved FRAP and NPNL motifs, which are essential for heme binding. Additionally, we examined *FurBox* sequences in the promoter regions to determine their role in iron-dependent regulation. Our findings confirm that these membrane proteins possess the necessary motifs to facilitate interaction with the heme group. Moreover, their *FurBox* sequences suggest that iron regulates their expression. In conclusion, these proteins represent promising therapeutic targets. Inhibiting them could reduce *P. aeruginosa* virulence by limiting iron uptake and biofilm formation, providing a foundation for future research on inhibitors targeting these proteins.

KEY WORDS: *P. aeruginosa*, heme, iron, protein, acquisition, pathogenicity.

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RESUMEN

Pseudomonas aeruginosa (*P. aeruginosa*) es una bacteria Gram negativa de gran relevancia clínica, responsable de infecciones nosocomiales graves, especialmente en pacientes inmunocomprometidos. Su capacidad para adquirir hierro es crucial para su patogenicidad, en este trabajo de investigación se buscó identificar proteínas de membrana clave involucradas en la captación del grupo hemo como posibles blancos terapéuticos. Para lograrlo, se realizó un análisis *in silico*, evaluando 47 proteínas de membrana potenciales mediante *ExPASy BLAST*. Se identificaron siete proteínas de membrana candidatas que contienen los motivos conservados FRAP y NPNL, esenciales para la unión al hemo. Además, se analizó la presencia de secuencias *FurBox* en las regiones promotoras para evaluar su regulación por hierro. Nuestros hallazgos muestran que las proteínas de membrana encontradas contienen los motivos necesarios para permitir su interacción con el grupo hemo. Además, presentan secuencias *FurBox*, sugiriendo que son reguladas por hierro. En conclusión, estas proteínas representan prometedores blancos terapéuticos; su inhibición podría reducir la virulencia de *P. aeruginosa* al limitar la adquisición de hierro y la formación de biopelículas, sentando las bases para futuros estudios sobre inhibidores dirigidos a estas proteínas.

PALABRAS CLAVE: *P. aeruginosa*, hemo, hierro, proteína, adquisición, patogenicidad.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative opportunistic bacterium widely known for its ability to cause nosocomial infections, particularly in immunocompromised patients, such as those with cystic fibrosis, severe burns, or those using invasive medical devices (Wunderink & Mendoza, 2007; Sathe *et al.,* 2023). Infections caused by *P. aeruginosa* account for up to 15 % of hospital-acquired infections worldwide (Alhazmi, 2015; Lopes *et al.,* 2022). This pathogen poses a significant challenge to healthcare systems due to its multidrug resistance and ability to form biofilms, providing it with a major advantage in clinical environments (Banin *et al*., 2005; Thi *et al*., 2020).

One of the most critical factors contributing to the pathogenicity of *P. aeruginosa* is its ability to acquire iron from its surroundings. Iron is an essential micronutrient for nearly all living organisms, playing a crucial role in vital metabolic processes such as DNA synthesis, energy production via cellular respiration, and defense against oxidative stress (Gozzelino & Arosio, 2015; Bradley *et al*., 2020). In the human body, iron is primarily stored in proteins such as hemoglobin,

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ferritin, lactoferrin, and hemosiderin (Sánchez *et al*., 1992; Page, 2019; Kontoghiorghes & Kontoghiorghe, 2020). This storage mechanism limits free iron, preventing its potential toxicity and the generation of reactive oxygen species through the Fenton reaction, which could cause cellular damage (Krewulak & Vogel, 2008; Xiao *et al*., 2024). Additionally, free heme groups can exhibit oxygenase or peroxidase activity, potentially damaging proteins and DNA (Anzaldi & Skaar, 2010; Vlasova, 2018). Thus, free iron is scarce, making its acquisition by bacteria particularly challenging (Wooldridge & Williams, 1993; Ullah & Lang, 2023).

Due to the limited availability of free iron in the host, many pathogenic bacteria, including *P. aeruginosa*, have developed highly specialized mechanisms for iron uptake (Hoffmann *et al*., 2001). *P. aeruginosa* can achieve this by producing siderophores (Budzikiewicz, 2001; Bonneau *et al*., 2020) or capturing exosiderophores (Perraud *et al*., 2020), molecules that extract iron from the environment. It can also acquire heme directly, a rich source of iron found in hemoglobin (Andrews *et al*., 2003; Becker & Skaar, 2014). The ability of *P. aeruginosa* to obtain iron not only allows it to survive in hostile environments but is also crucial for biofilm formation, one of the bacterium's primary strategies for evading the immune system and resisting antibiotic treatments (Alhazmi, 2015; Tuon *et al*., 2022).

Heme, an iron-containing complex, serves as a key iron source for many pathogens, including *P. aeruginosa* (Anzaldi & Skaar, 2010; Schalk & Parraud, 2023). In the human body, heme is found in proteins such as hemoglobin and myoglobin. During an infection, *P. aeruginosa* uses exotoxins and proteases to lyse host cells, releasing heme from these proteins (Reynolds & Kollef, 2021). This heme is subsequently captured by *TonB*-dependent outer membrane proteins (Krewulak & Vogel, 2008; Silale & Van den Berg, 2023). These proteins are part of three major heme uptake systems in *P. aeruginosa*: The Phu (*Pseudomonas heme uptake*) system, the Has (*heme assimilation system*), and the Hxu system (Ochsner *et al*., 2000; Otero-Asman *et al*., 2019; Normant *et al*., 2022).

The *Phu* system consists of the PhuR protein, which acts as a heme receptor, capturing and transporting it across the membrane (Ochsner *et al*., 2000). The *Has* system includes the hemophore HasAp, which sequesters heme and transfers it to an outer membrane receptor (Otero-Asman *et al*., 2019). Finally, the *Hxu* system contains the HxuA protein, which is expressed at high heme concentrations and is regulated by the *HxuI* and *HxuR* proteins, acting as sigma factors. While the Phu and Has systems are primarily active, *Hxu* serves as a heme sensor and plays a secondary role in heme acquisition (Yang *et al*., 2022). This redundancy in iron uptake capabilities makes *P. aeruginosa* highly adaptable to its environment (Ochsner *et al*., 2000; Otero-Asman *et al*., 2019; Normant *et al*., 2022).

Pathogenic bacteria can capture heme through outer membrane proteins containing the conserved FRAP and NPNL motifs, which are crucial for heme binding (Smith & Wilks, 2012). These motifs enable membrane proteins to interact with heme, facilitating its transport into the bacterial cell (Smith & Wilks, 2012; Marson *et al*., 2024). The arginine (R) residue in the FRAP motif forms ionic bonds or hydrogen bridges with the heme group, while the proline (P), asparagine (N), and leucine (L) residues in the NPNL motif stabilize the structure and enhance heme binding (Smith & Wilks, 2012).

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The regulation of these iron uptake systems is mediated by the Fur (Ferric uptake regulator) protein. In the presence of iron, Fur binds to consensus sequences known as *FurBox* within the promoter regions of iron-related genes, inhibiting their expression (Griggs *et al*., 1987; Escolar *et al*., 1999; Cornelis, 2010; Lee, 2023). However, when iron levels are low, *Fur* no longer represses these genes, allowing for the synthesis of proteins involved in iron uptake (Pasqua *et al*., 2017; Kang *et al*., 2024). This mechanism ensures that *P. aeruginosa* can rapidly adapt to changes in iron availability in its environment, a crucial factor for its survival and virulence during infections (Cornelis, 2010; Pasqua *et al*., 2017).

Given the importance of iron in *P. aeruginosa* pathogenicity, identifying key proteins involved in iron uptake is of great interest for developing new therapeutic approaches (Hurdle *et al*., 2011; Candel *et al*., 2022). Inhibiting these proteins could limit the bacterium's ability to acquire iron, thereby reducing its virulence and capacity to form *biofilms* (Banin *et al*., 2005; Wißbrock *et al*., 2019). Previous studies have shown that proteins containing FRAP and NPNL motifs facilitate heme interaction and transport (Louvel *et al*., 2006; Wißbrock *et al*., 2019; Liu *et al*., 2021). Therefore, this study conducted an *in silico* analysis of the *P. aeruginosa* proteome to identify proteins with these conserved motifs, using bioinformatics tools such as *ExPASy BLAST*, *Clustal Omega*, and *Swiss-Model* (Paul *et al*., 2020).

Material and Methods

Search for conserved sequences

The objective was to identify *P. aeruginosa* proteins with amino acid sequences similar to those that can bind the heme group, which is a key process in bacterial iron acquisition. To achieve this, a search was conducted in the *ExPASy BLAST* database (https://www.uniprot.org/blast/), using the sequences of ChuA from *E. coli* (Q7DB97_ECO57) and FrpB2 from *H. pylori* (Q9ZKT4_HELPJ) as controls, given that their ability to bind heme has been experimentally confirmed. Only sequences with an identity percentage greater than 30 % were selected (Pearson, 2013). The search was limited to the *P. aeruginosa* PAO1 proteome.

Alignment and analysis of conserved motifs

To confirm the presence of the FRAP and NPNL motifs, the obtained sequences were aligned to identify these motifs using the *Clustal Omega* server (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). Following this, the sequences were examined with the *KEGG Motif* server (https://www.genome.jp/tools/motif/) to detect conserved regions characteristic of proteins belonging to the *TonB* family.

Identification of key amino acid residues

The objective was to identify histidine (H), tyrosine (Y), and cysteine (C) residues that may be involved in heme binding, complementing the identification of the FRAP and NPNL

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motifs. For this purpose, the obtained sequences were analyzed using the *HemoQuest* server (http://131.220.139.55/SeqDHBM/), which allows for the identification of these residues in selected proteins.

Structural modeling of selected proteins

To analyze the spatial and three-dimensional conformation of the selected proteins, we modeled them using the *Swiss-Model* server (https://swissmodel.expasy.org/interactive). This allowed us to verify the locations of the FRAP and NPNL motifs, as well as the H, Y, or C residues within the three-dimensional structure of the proteins. The resulting models were processed with *Chimera* software (v1.18), enabling detailed visualization of their 3D structure and the positioning of the motifs and amino acids needed for heme binding.

Identification of coding sequences and promoter region analysis

The purpose was to determine whether the genes encoding these proteins are regulated by the *Fur* system, which would indicate a response to iron availability in the environment. To achieve this, the nucleotide sequences corresponding to the proteins were retrieved from the *KEGG* database (https://www.genome.jp/kegg/), selecting 100 nucleotides upstream of each sequence. These promoter regions were analyzed to identify the *FurBox* consensus sequence (5'-GATAATGATAATCATTATC-3').

Visualization and representation of conserved motifs

To visualize amino acid and nucleotide conservation patterns and highlight key positions within the sequences, the data were processed using *JalView* (v2.11.3.3). A graphical representation of conserved motifs was generated using the *WebLogo* server (https://weblogo.berkeley.edu/logo.cgi) (Crooks *et al*., 2004). This graphical analysis allowed for the identification of the most conserved regions within the selected sequences.

Data analysis

Each bioinformatics platform used in this study provides statistically significant results based on its algorithm. For instance, the *BLAST* program compares protein sequences and calculates the statistical significance of matches. Alignments performed with *Clustal Omega* utilize its HMM algorithm, which predicts probabilistic models and determines sequence relationships. Similarly, most of the bioinformatics servers employed in this research operate using statistically robust algorithms. Therefore, our data analysis was descriptive, rigorously following the parameters established by each platform to ensure that the obtained results were meaningful.

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Results and Discussion

The present study focused on the identification of key proteins involved in heme uptake by *P. aeruginosa* using an *in silico* approach.

Membrane proteins capable of heme binding share several common features: they are *TonB-dependent proteins*, contain the FRAP and NPNL motifs, as well as H, Y, or C residues; they are iron-regulated and structurally exhibit exposed motifs to enable interaction with the heme group.

The analysis allowed us to identify seven proteins with characteristics suggesting their potential involvement in iron uptake through heme binding.

P. aeruginosa possesses several hypothetical heme-binding proteins

To identify proteins with the ability to bind heme groups, a search was conducted in the *P. aeruginosa* PAO1 proteome using *ExPASy BLAST.*

A total of 47 sequences were obtained, with identity values ranging from 22.4 % to 99 %. When applying the *BLAST* algorithm, the selection criteria for further analysis included proteins with identity greater than 30 %, bit score above 50, and *E-value* < 10^{-6} compared to the reference proteins (Pearson, 2013) (Table 1).

The cutoff values for selecting proteins with potential heme-binding capacity were based on the *BLAST* algorithm. Under this premise, the selected proteins demonstrated high identity to the reference proteins. These values are considered statistically significant (Pearson, 2013). A total of twenty-one proteins met these criteria.

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Table 1. Obtained sequences.

Sequences producing significant alignments	Bits	Score E
TR:Q9HV88 Q9HV88_PSEAE Heme/hemoglobin uptake outer membrane recep...	182	1e-49
TR:Q9HYJ7 Q9HYJ7_PSEAE Heme uptake outer membrane receptor HasR OS...	145	9e-37
TR:Q9I442 Q9I442_PSEAE Probable heme utilization protein OS=Pseudo...	142	8e-36
TR:Q9I2I2 Q9I2I2_PSEAE Probable TonB-dependent receptor OS=Pseudom...	114	5e-27
TR:Q9I473 Q9I473_PSEAE Probable tonB-dependent receptor OS=Pseudom...	91.3	8e-20
SP:Q9I527 PIRA_PSEAE Ferric enterobactin receptor PirA OS=Pseudomo...	84.3	2e-17
TR:Q9I3X9 Q9I3X9_PSEAE Probable siderophore receptor OS=Pseudomona...	82.8	5e-17
SP:Q05098 PFEA_PSEAE Ferric enterobactin receptor OS=Pseudomonas a...	81.3	1e-16
TR:Q9HUR6 Q9HUR6_PSEAE Secretin/TonB short N-terminal domain-conta...	71.2	2e-13
TR:Q9I1I6 Q9I1I6_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	69.7	6e-13
TR:Q9I116 Q9I116_PSEAE Ferrioxamine receptor FoxA OS=Pseudomonas a...	69.3	8e-13
TR:Q9HVC0 Q9HVC0_PSEAE Probable TonB-dependent receptor OS=Pseudom...	67.8	2e-12
TR:Q9HZT6 Q9HZT6_PSEAE Probable TonB-dependent receptor OS=Pseudom...	63.5	5e-11
TR:Q9HYX3 Q9HYX3_PSEAE Probable TonB-dependent receptor OS=Pseudom...	57.0	5e-09
TR:Q9HXB2 Q9HXB2_PSEAE Fe(III) dicitrate transport protein FecA OS...	55.5	2e-08
TR:Q9I5F7 Q9I5F7_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	55.1	2e-08
TR:Q9I648 Q9I648_PSEAE Ferrichrome receptor FiuA OS=Pseudomonas ae...	54.7	3e-08
TR:Q9I0P7 Q9I0P7_PSEAE Secretin/TonB short N-terminal domain-conta...	53.9	5e-08
TR:Q9I6Y1 Q9I6Y1_PSEAE Probable TonB-dependent receptor OS=Pseudom...	53.1	8e-08
TR:Q9I3A9 Q9I3A9_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	51.6	2e-07
TR:Q9I226 Q9I226_PSEAE Secretin/TonB short N-terminal domain-conta...	51.6	2e-07
TR:Q9I422 Q9I422_PSEAE Probable TonB-dependent receptor OS=Pseudom...	49.7	9e-07
SP:P48632 FPVA_PSEAE Ferripyoverdine receptor OS=Pseudomonas aerug...	49.7	9e-07
TR:Q9I683 Q9I683_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	48.1	3e-06
TR:Q9I245 Q9I245_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	47.8	4e-06
TR:Q9I258 Q9I258_PSEAE TonB-dependent receptor OS=Pseudomonas aeru...	46.6	9e-06
TR:G3XD89 G3XD89_PSEAE Copper transport outer membrane porin OprC ...	44.7	3e-05
TR:Q9I1E3 Q9I1E3_PSEAE Probable TonB-dependent receptor OS=Pseudom...	40.4	7e-04
TR:Q9HWL3 Q9HWL3_PSEAE Second ferric pyoverdine receptor FpvB OS=P...	40.4	7e-04
SP:Q9HUX3 CNTO_PSEAE Metal-pseudopaline receptor CntO OS=Pseudomon...	38.9	0.002
SP:A0A0H2ZI93 CNTO_PSEAB Metal-pseudopaline receptor CntO OS=Pseud...	38.5	0.003
SP:G3XCY8 PIUA_PSEAE Probable TonB-dependent siderophore receptor ...	36.6	0.010
TR:Q9I6U2 Q9I6U2_PSEAE Probable TonB-dependent receptor OS=Pseudom...	36.6	0.010
SP:P42512 FPTA_PSEAE Fe(3+)-pyochelin receptor OS=Pseudomonas aeru...	35.8	0.017
TR:Q9I2J4 Q9I2J4_PSEAE Ferric-mycobactin receptor, FemA OS=Pseudom...	35.4	0.023
TR:Q9I0X7 Q9I0X7_PSEAE Tyrosine porin OpdT OS=Pseudomonas aerugino...	30.8	0.55
SP:Q02PW7 IHFB_PSEAB Integration host factor subunit beta OS=Pseud...	26.6	3.6

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Continuation

Table 1. Obtained sequences.

Sequences producing significant alignments	Bits	Score E
SP:B7VAL8 IHFB_PSEA8 Integration host factor subunit beta OS=Pseud...	26.6	3.6
SP:A6V2R4 IHFB_PSEA7 Integration host factor subunit beta OS=Pseud...	26.6	3.6
SP:Q51473 IHFB_PSEAE Integration host factor subunit beta OS=Pseud...	26.6	3.6
TR:Q9HWM5 Q9HWM5_PSEAE Probable TonB-dependent receptor OS=Pseudom...	27.7	5.4
TR:Q9I3A5 Q9I3A5_PSEAE Probable AMP-binding enzyme OS=Pseudomonas ...	27.3	6.9
TR:G3XD90 G3XD90_PSEAE MotD OS=Pseudomonas aeruginosa (strain ATCC...	26.9	7.7
SP:Q02F80 PURA_PSEAB Adenylosuccinate synthetase OS=Pseudomonas ae...	26.9	8.6
SP:B7V201 PURA_PSEA8 Adenylosuccinate synthetase OS=Pseudomonas ae...	26.9	8.6
SP:Q9HUM6 PURA_PSEAE Adenylosuccinate synthetase OS=Pseudomonas ae...	26.9	8.6
TR:Q9HWU4 Q9HWU4_PSEAE Usher CupB3 OS=Pseudomonas aeruginosa (stra...	26.9	9.6

Using the sequences *ChuA* from *E. coli* and *FrpB2* from *H. pylori* as reference, 47 sequences were identified in the proteome of *P. aeruginosa* PAO1, the obtained bits and E-values (Score E) are shown.

Source: Authors' own work.

P. aeruginosa protein sequences identified are TonB-dependent

The identified sequences have hypothetical names based on their identity with the reference sequences used. In general, they include proteins classified as "*Heme/hemoglobin uptake*," "*Probable TonB-dependent receptor*," "*TonB-dependent receptor*," "*Ferric enterobactin receptor*," and "*Probable siderophore receptor*," among others (Table 1).

To refine these findings, a more detailed analysis of the twenty-one identified proteins was conducted to determine whether they belong to the *TonB-dependent* protein family. For this purpose, the obtained amino acid sequences were analyzed using the *KEGG Motif* server.

The results confirm that all twenty-one proteins belong to the *TonB-dependent* family (Table 2). These proteins are essential for iron uptake through heme binding (Balhesteros *et al*., 2017; Schalk & Perraud, 2023). They have a β-barrel structure composed of antiparallel β-sheets, which create a highly selective channel for molecules such as heme, ensuring its efficient transport into the bacterium (Noinaj *et al*., 2010). In addition to their transport function, this structural configuration provides stability to the bacterial outer membrane, allowing adaptation to hostile environments (Sajeev-Sheeja *et al*., 2023). Inside the β-barrel, an internal *plug* regulates molecule passage by blocking the channel in the absence of a specific substrate. This *plug* contains specific sites, including H, Y, and C residues, which directly interact with the substrate, ensuring transport specificity (Noinaj *et al*., 2013).

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Transport through *TonB-dependent* proteins involves a mechanical interaction mechanism. When the ligand binds to the *plug*, a shearing or traction mechanism is triggered, disrupting networks of electrostatic interactions on the inner surface of the β-barrel, allowing for crucial conformational changes (Mouriño & Wilks, 2021). The binding of the ligand to the extracellular side of the *plug* extends the *TonB* box in the periplasmic region, facilitating interaction with the *TonB* system (Mouriño & Wilks, 2021). This system, which harnesses energy from the proton-motive force, temporarily unlocks the *plug*, enabling the heme group to pass into the periplasmic space (Mey & Payne, 2001; Noinaj *et al*., 2013).

This integrated process is crucial for bacteria to transport iron from heme groups into the cell. Heme signaling and transport require a precise interaction between *TonB-dependent* receptors and outer membrane proteins, underscoring the importance of their structural characteristics (Sheldon *et al*., 2016).

In addition to being *TonB-dependent*, the twenty-one identified proteins exhibit a β-barrel structure and possess an internal *plug* (Table 2). Similarly, the control proteins used in this study also exhibit this structural organization and *plug* domain.

Table 2. Motifs present in localized proteins.

Protein	Motifs
*P. aeruginosa*_phuR	M-TonB, M-β, M-plug
*P. aeruginosa*_HasR	M-TonB, M-β, M-plug, M, S-TonB
*P. aeruginosa*_Hxu	M-TonB, M-β, M-plug, M, S-TonB, F-P
*P. aeruginosa*_Q9I2I2	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I473	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I527	M-TonB, M-β, M-plug, F-L
*P. aeruginosa*_Q9I3X9	M-TonB, M-β, M-plug
*P. aeruginosa*_Q05098	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9HUR6	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I1I6	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I116	M-TonB, M-β, M-plug, SpF
*P. aeruginosa*_Q9HVC0	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9HZT6	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9HYX3	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9HXB2	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I5F7	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I648	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I0P7	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I6Y1	M-TonB, M-β, M-plug, T3SS

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Table 2. Motifs present in localized proteins.

Protein	Motifs
*P. aeruginosa*_Q9I3A9	M-TonB, M-β, M-plug
*P. aeruginosa*_Q9I226	M-TonB, M-β, M-plug
*H. pylori*_FrpB2	M-TonB, M-β, M-plug
*E. coli*_ChuA	M-TonB, M-β, M-plug

Sequence analysis revealed the presence of domains such as: *TonB* receptor motif (M-*TonB*), β-barrel structure (S-β), *Plug* motif (M-plug), *TonB* receptor and secretin motif (M, S-*TonB*), Peptidase family (F-P), Laterosporulin family (F-L), F-pilus system (SpF), and Type III Secretion System (T3SS).

Source: Authors' own work.

The identified *P. aeruginosa* proteins contain heme-binding motifs

Not all *TonB-dependent* proteins are necessarily capable of binding heme; they must also contain specific motifs such as FRAP and NPNL. To confirm the presence of these motifs, an alignment of the twenty-one selected proteins was performed using *Clustal Omega.*

As a result, seven proteins were found to conserve the FRAP and NPNL motifs or their variants (Q9HV88 (PhuR), Q9HYJ7 (HasR), Q9I442 (Hxu), Q9I2I2, Q9I527, Q05098, Q9I473), (Figure 1). It is noteworthy that among the identified proteins, PhuR, HasR, and Hxu have previously been characterized as heme-binding proteins (Ochsner *et al*., 2000; Otero-Asman *et al*., 2019; Kümmerli, 2023), this supports the validity of our methodology in identifying proteins with this capacity.

While the conservation of the FRAP and NPNL motifs is important for heme binding, it has been documented that certain amino acid variations in these motifs do not completely eliminate heme-binding capacity (Bracken *et al*., 1999; Nienaber *et al*., 2001; Murphy *et al*., 2002; Louvel *et al*., 2006; Liu *et al*., 2021). Therefore, the presence of these motifs in the seven selected proteins strongly suggests their potential ability to bind heme.

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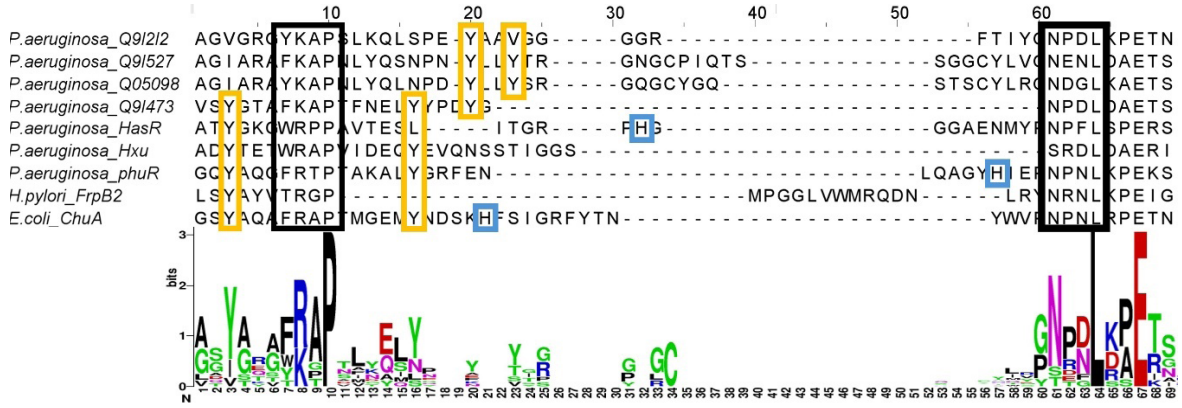

Figure 1. Alignment of FRAP and NPNL consensus sequences.

Proteins from the *P. aeruginosa* proteome were aligned, along with reference sequences from *E. coli* and *H. pylori*, to identify FRAP and NPNL motifs, as well as H and Y residues potentially involved in heme group binding. The sequence logo is shown, where the larger letters represent the better conserved residues. On the left, the name of the bacterial strain is followed by the number or name of the protein analyzed. The image shows a fragment of the complete protein sequence.

Source: Authors' own work.

In a study performed by our research group we demonstrated that the *FrpB2* protein of *H. pylori* is able to bind the heme group contained in hemoglobin. It can be seen that the FRAP and NPNL motifs have small variations (Figure 1), but in spite of this it retains this binding capacity (Mey & Payne, 2001; González-López & Olivares-Trejo, 2009; Liu *et al*., 2021).

Conversely, some authors suggest that the presence of a H between the two motifs enhances heme binding (Hare, 2017), as noted in the *HemR* protein of *Y. enterocolitica* (Bracken *et al*., 1999). Nevertheless, the *FrpB2* protein in *H. pylori* lacks this H but still maintains its binding capacity (González-López & Olivares-Trejo, 2009). Furthermore, other researchers have shown that heme binding is not solely reliant on a single H; other amino acids, such as Y (Allen & Schmitt, 2011; Draganova *et al*., 2015) and C (Wißbrock *et al*., 2019), can also contribute to this process. In light of this, we conducted an analysis using the *HemoQuest* server to identify H, Y, or C residues that may have potential heme-binding capabilities in the selected sequences.

Among the seven candidate proteins, both H and Y residues capable of binding heme were identified (Table 3). *HemoQuest* estimates binding capacity based on the dissociation constant (K_D), which is derived from experimental structural data and biophysical models considering solvent accessibility and the local polarity of the heme-binding site (Chen & Zhou, 2005). Heme-binding affinities are predicted by comparing sequences with profiles of known proteins, refined through computational simulations and molecular docking (Paul *et al*., 2020). Furthermore, the residues must possess a positive charge to facilitate interaction with the heme group (Wißbrock *et al*., 2019).

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Table 3. Amino acids with the ability to bind the heme group.

Bacteria-protein	Residue	Net chargue	K_D (μM)
P. aeruginosa_Q9I2I2	H313	+3	1.16
	H415	+1	11.51
	C497	+1	1.91
	C501	+2	0.36
P. aeruginosa_Q05098	Y335	+1	3.8
	Y664	+3	2.53
	Y666	+2	7.48
	H667	+1	12.31
P. aeruginosa_HasR	H348	+3	2.31
	H624	+2	15.77
H. pylori_FrpB2	Y558	+1	2.7
	H737	+3	9.55
	H739	+3	1.03
	Y756	+4	0.44
P. aeruginosa_Q9I527	Y2	+1	13.92
	Y319	+1	3.74
	H667	+2	1.47
P. aeruginosa_Q9I473	H247	+1	16.21
	H442	+2	8.67
P. aeruginosa_Hxu	H553	+2	3.17
	Y555	+1	6.77
	Y560	+1	5.29
	H617	+2	0.53
	H718	+1	0.47
P. aeruginosa_phuR	H484	+3	3.11
	Y564	+2	2.21
	Y628	+2	12.3
E. coli_ChuA	H353	+1	0.53
	H448	+1	0.14
	H596	+1	5.15

The table shows the amino acids and their position within the protein with the ability to bind to the heme group, their net charge is shown, as well as their dissociation constant (K_D).

Source: Authors' own work.

On the other hand, an H residue between the FRAP and NPNL motifs was observed in only two of the seven proteins (HasR and PhuR). The other candidate proteins identified through the *HemoQuest* analysis contain Y residues that can bind the heme group (Table 3). Interestingly, the proteins Q9I2I2, Q9I527, Q9I473, and Q05098 possess a Y residue between these motifs. The Hxu protein, previously characterized *in vitro* for its heme-binding capacity, does not retain the central H but does have a Y residue present between these motifs (Otero-Asman *et al*., 2019; Yang *et al*., 2022) (Figure 1).

Hence, the candidate proteins possess the necessary motifs and residues to bind the heme group. Additionally, the control proteins used in the study display similar characteristics, further supporting their potential role in iron acquisition from heme groups.

The heme-binding motifs are exposed

The FRAP and NPNL motifs, as well as the H and/or Y residues, must be spatially exposed

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to interact with the heme group. To analyze the three-dimensional arrangement of these amino acids, protein modeling was performed.

Using the *Swiss-Model* server, we obtained the three-dimensional structures of the seven candidate proteins. Upon examining these structures, we confirmed that the FRAP and NPNL motifs, along with the key residues identified by *HemoQuest*, are exposed (Figure 2).

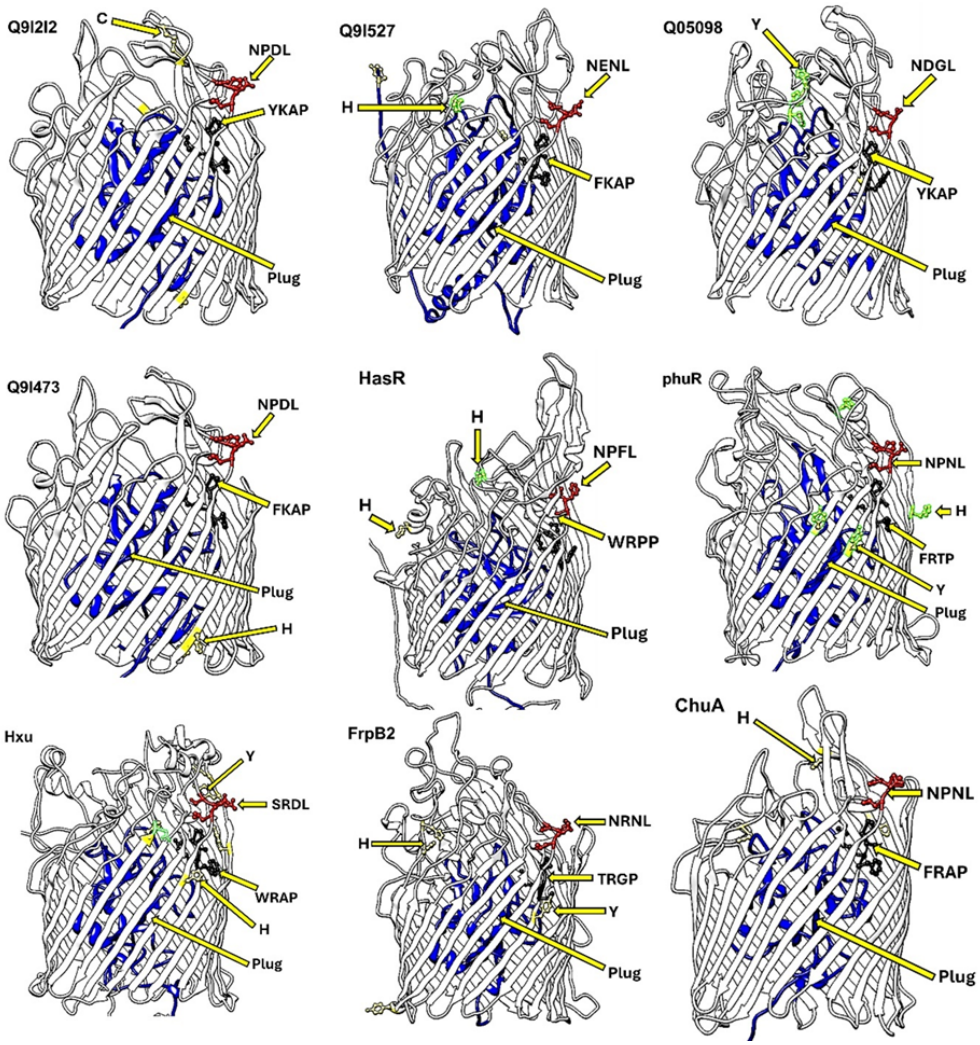

Figure 2. Protein modeling.

Modeling of both identified proteins and reference sequences (*ChuA* from *E. coli* and *FrpB2* from *H. pylori*) is shown to observe their spatial structures. In all models, the conserved FRAP and NPNL motifs are located at the top and are exposed on the outer surface of the protein. All proteins present a barrel-shaped structure β, the *plug* structure is shown in blue, the FRAP motif and its variants in black, the NPNL motif and its variants in red, and the H and Y residues are shown in yellow.

Source: Authors' own work.

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It is evident that the interaction with the heme group requires a coordinated interplay between the FRAP and NPNL motifs and the H, Y, or C residues throughout the protein (Mayfield *et al*., 2011; Smith & Wilks, 2012; Brewitz *et al*., 2017; Naoe *et al*., 2017; Marson *et al*., 2024).

The *FurBox* consensus sequence is located in the promoter region

An additional criterion for selecting candidate heme-binding proteins was to determine whether their expression is regulated by iron availability. Proteins regulated by iron typically have the *FurBox* consensus sequence in their promoter region (Escolar *et al*., 1999; Fillat, 2014; Kang *et al*., 2024).

After analyzing the promoter regions, we found that the seven coding genes contain the *FurBox* sequence in this region (Figure 3). The predominant nucleotides in this region should be adenine and thymine (Fillat, 2014). While we observed variations in similarity to the consensus sequence, the adenine-thymine content was never lower than 50 %, suggesting that the regulation of these proteins may be differential and dependent on iron availability in the environment (Ochsner *et al*., 2000; Fillat, 2014).

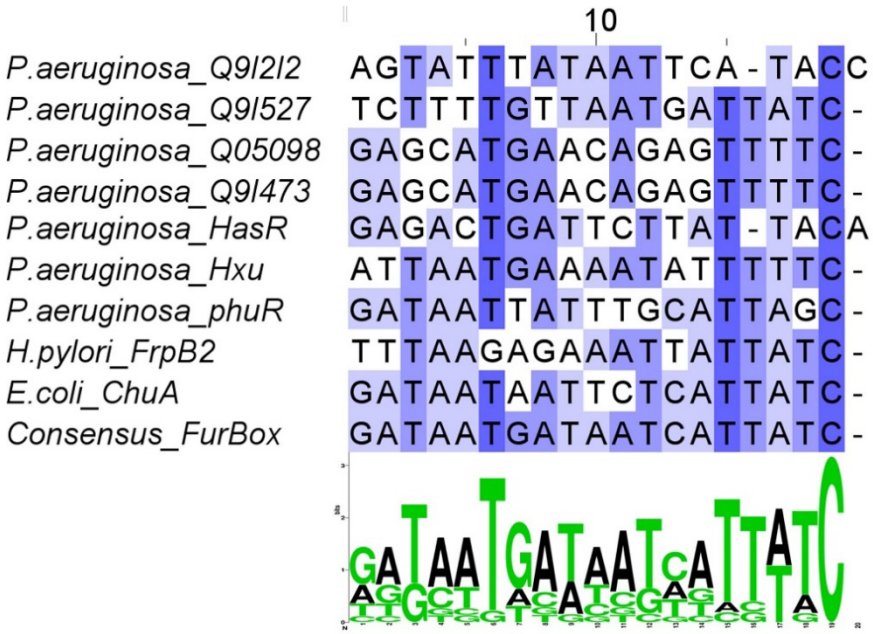

Figure 3. *FurBox* sequence alignment.

The FurBox consensus sequence is shown, highlighting its conservation in different promoter sequences encoding localized proteins. At the bottom of the figure, the sequence logo shows the conserved nucleotides; the larger letters represent the most conserved nucleotides. The image shows only a fragment of the complete nucleotide sequence.

Source: Authors' own work.

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The identification of *FurBox* sequences in promoter regions reinforces the hypothesis that these proteins play a crucial role in *P. aeruginosa*'s response to iron deficiency. This mechanism ensures that the bacterium expresses only the genes required for iron uptake when iron is scarce, reflecting *P. aeruginosa*'s adaptability to different iron sources (Pasqua *et al*., 2017).

Iron uptake is critical for *P. aeruginosa* pathogenesis, particularly in biofilm formation (Banin *et al*., 2005; Zhang *et al*., 2021), which is associated with multidrug-resistant strains (Alhazmi, 2015) that cause infections in hospitalized patients, especially those with cystic fibrosis and pulmonary diseases (Tuon *et al*., 2022). Restricting this bacterium's iron access could significantly reduce these infections and make it more susceptible to conventional antibiotics, thereby decreasing the evolutionary pressure for resistance (Post *et al*., 2019; Jiang *et al*., 2020).

Our study represents a significant step forward in combating *P. aeruginosa*. By identifying key proteins involved in iron uptake, we have uncovered potential therapeutic targets to counteract its pathogenicity. Inhibiting these proteins could reduce both virulence and survival, as it would disrupt an essential process for bacterial growth (Rosas & Lithgow, 2022; Sánchez-Jiménez *et al*., 2023).

The existence of multiple heme-binding proteins expands the therapeutic options (Hamad *et al*., 2022). Metallotherapeutics, initially developed for cancer treatment, have gained relevance in the antimicrobial field due to the rise of drug-resistant bacterial strains. Metal-based compounds can disrupt essential metal-utilization pathways in bacteria, compromising their survival and virulence (Centola *et al*., 2020).

For instance, *P. gingivalis* cannot synthesize protoporphyrin IX, which is essential for its metabolism, and must acquire it from the environment. Heme, which contains protoporphyrin IX, is internalized through the HA2 membrane receptor. Based on this knowledge, researchers designed a deuteroporphyrin-metronidazole conjugate, known as a "Trojan horse" approach. This conjugate showed significantly greater efficacy against *P. gingivalis* than gentamicin and metronidazole. A 4-hour incubation with deuteroporphyrin-metronidazole at a 40 $\mu mol \cdot L^{-1}$ concentration completely eliminated *P. gingivalis* (Ye *et al*., 2017).

Another research group designed a porphyrin-nitroimidazole conjugate against *P. gingivalis*, demonstrating similar efficacy to metronidazole at 4 μM. The authors stated that this conjugate was designed based on interactions with amino acids in the H2A protein (Dingsdag *et al*., 2015).

Although the coexistence of multiple iron and heme uptake pathways poses a challenge for long-term therapeutic success, these pathways represent promising targets for innovative antimicrobial strategies. Leveraging the different bacterial iron uptake systems for targeted antibiotic delivery could lead to more effective treatments.

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Study limitations and future perspectives

The *in silico* analysis provides a strong foundation for identifying key proteins in heme acquisition, but it is essential to perform experimental validations to confirm their functionality. *In vitro* studies should assess these proteins' heme-binding capabilities and their potential as therapeutic targets. Additionally, testing specific inhibitors in animal infection models could complement current treatments, particularly against multidrug-resistant *P. aeruginosa* strains.

Conclusions

This *in silico* study identified seven membrane proteins in *P. aeruginosa* containing the conserved FRAP and NPNL motifs, which are essential for iron acquisition via heme binding. Among these proteins, PhuR, HasR, and Hxu emerge as promising therapeutic targets. Their inhibition could reduce bacterial virulence, impairing its ability to form biofilms and colonize the host.

The analysis revealed *FurBox* sequences in the genes encoding these proteins, indicating that their expression is regulated by iron availability. Structural modeling confirmed the exposure of FRAP and NPNL motifs on the protein surface, facilitating interaction with the heme group, while *HemoQuest* identified additional key residues involved in iron uptake.

This study provides a solid foundation for forthcoming *in vitro* assays, suggesting that targeting iron acquisition in *P. aeruginosa* could be an effective strategy against multidrug-resistant infections.

A short-term perspective is to verify the functionality of these proteins through genetic engineering-based assays in *in vitro* experiments.

Authors' contributions

Work conceptualization, MAGL; methodology development, EMGC, MAGL; software management, MAGL, JJOT; analysis of results, MAGL, EMGC, and JJOT; data management, MAGL, EMGC, and JJOT; manuscript writing and preparation, MAGL, EMGC, and JJOT; drafting, revising and editing MAGL, EMGC, and JJOT; fund acquisition, MAGL, EMGC.

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Conflict of interest

The authors declare that they have no conflict of interest

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