Salomé Esteves Mota
Bachelor of Science in Biotechnology

Tetrapyrrole Metabolism of *Staphylococcus aureus*

Dissertation for the obtainment of a M.Sc. in Biochemistry for Health

Supervisor: Marco Videira PhD, Post-doc, ITQB-UNL
Co-supervisor: Lígia M. Saraiva PhD, Principal Investigator, ITQB-UNL

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Salomé Esteves Mota
Instituto de Tecnologia Química e Biológica António Xavier (ITQB-NOVA),
Universidade Nova de Lisboa (UNL).

Esta dissertação é dedicada à minha família, em especial aos meus pais e avós, namorado e amigos que sempre me apoiaram e me deram força e motivação para continuar mais uma etapa da minha vida.

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Abstract

Staphylococcus aureus is a commensal bacterium with its pathogenicity associated with toxin-mediated diseases, several infections, and the presence of iron-containing tetrapyrroles, Haem and Sirohaem which are crucial for survival within the human host enabling infection.

Haem can be synthesised endogenously or acquired from the environment by haem uptake systems being essential for many bioprocesses. It can be synthesised via three different pathways: protoporphyrin-dependent pathway, sirohaem-dependent pathway, and coproporphyrin-dependent pathway. Sirohaem is involved in sulphate and nitrate reduction as a cofactor for sulphite and nitrite reductases, synthesised either by one, two or three enzymes: sirohaem synthase CysG (e.g., present in *E. coli*), *met1* and *met8* genes (e.g., present in fungi and bacteria) and the proteins UroM, P2D and ShfC (e.g., present in *S. aureus*).

To understand how the absence of specific genes can affect the biosynthesis of haem, the Alkaline haematin D-575 and the Pyridine hemochrome method were used to quantify the levels of haem in *S. aureus* strains (NCTC, Newman, Δ*CpfC*, Δ*ChdC*, Δ*hmp*, Δ*IsdG*, Δ*IsdC*, Δ*hrtB* and Δ*htsA*). One of the differences between these methods was the levels of haem in the Δ*hmp* mutant leading to inconclusive results concerning a possible correlation between the Hmp protein and the haem biosynthesis.

To study the effect of *S. aureus* sirohaem biosynthesis genes we complemented an *E. coli* strain unable to produce sirohaem with *uroM, p2D* and *shfC* genes of *S. aureus* and the products obtained were confirmed by mass spectroscopy. The impact in nitrate and sulphate assimilation was analysed in *S. aureus* strains (JE2, Δ*p2D*, Δ*shfC*, Δ*nirB* and Δ*nirD*) lacking sirohaem biosynthesis with different sulphur and nitrogen sources. No different phenotypes were found therefore, the nitrite consumption of *S. aureus* mutant strains (JE2, Δ*uroM,* Δ*p2D*, Δ*shfC* and Δ*nirB*) was evaluated in anaerobic conditions with nitrite. In conclusion, as haem and sirohaem are important for *S. aureus* growth, more studies are needed to understand their role and their importance for pathogens.

Keywords: *Staphylococcus aureus*, biosynthesis, haem, sirohaem, nitrite consumption

Resumo

Staphylococcus aureus é uma bactéria comensal com a sua patogenicidade associada a doenças mediadas por toxinas, diversas infeções, e a presença de tetrapirróis que contêm ferro, Hemo e Sirohemo, cruciais para a sobrevivência no hospedeiro permitindo a infeção.

O hemo pode ser sintetizado endogenamente ou adquirido do ambiente por sistemas de captação de hemo sendo essencial para muitos bioprocessos. Pode ser sintetizado por três vias diferentes: via dependente de protoporfirina, via dependente de sirohemo e via dependente de coproporfirina. O sirohemo está envolvido na redução de sulfato e nitrato como um cofator para sulfito e nitrito redutases, podendo ser sintetizado por uma, duas ou três enzimas: sirohemo sintase CysG (presente em *E. coli*), genes *met1* e *met8* (presentes em fungos e bactérias) e as proteínas UroM, P2D e ShfC (presentes em *S. aureus*).

Para entender como a ausência de genes específicos podem afetar a biossíntese do hemo, os métodos Alkaline haematin D-575 e Pyridine hemochrome foram usados para quantificar os níveis de hemo em estirpes de *S.aureus* (NCTC, Newman, *ΔCpfC, ΔChdC, Δhmp, ΔIsdG, ΔIsdC, ΔhrtB e ΔhtsA*). Uma das diferenças entre estes métodos foram os níveis de hemo obtidos na estirpe mutante *Δhmp*, levando a resultados inconclusivos sobre uma possível correlação entre a proteína Hmp e a biosíntesse do hemo.

Para estudar o efeito dos genes da biossíntese do sirohemo de *S. aureus*, foi complementada uma estirpe de *E. coli* incapaz de produzir sirohemo com os genes *uroM, p2D* e *shfC* de *S. aureus* e os produtos obtidos foram confirmados por espectroscopia de massa. O impacto na assimilação de nitrato e sulfato foi analisado em estirpes de *S. aureus* (JE2, *Δp2D, ΔshfC, ΔnirB* e *ΔnirD),* incapazes de produzir sirohemo, com diferentes fontes de enxofre e azoto. Não foram encontrados diferentes fenótipos, portanto, o consumo de nitrito de estirpes de *S. aureus* foi avaliado em condições anaeróbicas e na presença de nitrito. Em conclusão, o hemo e o sirohemo são importantes para o crescimento de *S. aureus,* e mais estudos são necessários para entender a sua importância.

Keywords: *Staphylococcus aureus*, biosíntese, hemo, sirohemo, consume de nitritos

Index

Figure Index

Table Index

Abbreviations, Acronyms and Symbols

ABC transporter – ATP binding cassette transporter;

agr – Accessory gene regulator*;*

AHD reagent – Alkaline Hematin D (detergent);

AIP – Auto inducing peptide;

BCA – Bicinchoninic assay acid;

BSA – Albumin Standard;

$CaCl_2$ – Calcium chloride;

CA-MRSA - Community-associated MRSA;

CHIP – Chemotaxis inhibitory protein;

Co^{2+} - Cobalt (2+);

CPD – Coproporphyrin-dependent pathway;

Cu^{1+} / Cu^{2+} - Copper (1+) / Copper (2+);

dH_2O – Distilled water;

DNA - Deoxyribonucleic acid;

E. coli - *Escherichia coli;*

Eap - Extracellular adherence protein;

FAD - Flavin adenine dinucleotide;

Fe^{2+} - Iron (2+);

H_2O_2 – Hydrogen peroxide;

HCl – Hydrochloric acid;

HPLC – Hight Performance Liquid Chromatography;

IPTG - Isopropyl β-D-1-thiogalactopyranoside;

Isd - Iron-regulated surface determinant;

$K_3Fe(CN)_6$ – Potassium ferricianide;

LA - Luria-Bertani agar;

LB - Luria-Bertani;

M9 - M9 minimal medium;

Mg^{2+} - Magnesium (2+);

MRSA - Methicillin-resistant *S. aureus;*

MS – Mass Spectrometry;

MSCRAMM - Microbial surface components recognizing adhesive matrix molecules;

N_2 – Nitrogen;

NaCl – Sodium chloride;

$NaNO_2$ – Sodium nitrite;

NaOH – Sodium hydroxide;

Ni^{2+} - Nickel (2+);

NO – Nitric oxide;

O_2 – Oxygen;

OD - Optical density;

PBS - Phosphate-buffered saline;

PCR – Polymerase Chain Reaction;

PPD – Protoporphyrin-dependent pathway;

Psi – Pound-force per square inch;

RNA - Ribonucleic acid;

Rpm - Rotations per minute;

RT – Room temperature;

S. aureus – *Staphylococcus aureus;*

SAM - S-adenosyl-L-methionine;

SHD – Sirohaem-depented pathway;

TFA – Trifluoroacetic acid;

TSA - Tryptic soy agar;

TSB - Tryptic soy broth;

UV – Ultraviolet;

VISA - Vancomycin-intermediate resistant *S. aureus;*

VRSA - Vancomycin resistant *S. aureus;*

WR – Working reagent;

Zn^{2+} - Zinc (2+);

δ-ALA - δ-aminolaevulinic acid;

1. Introduction

1.1. *Staphylococcus aureus*

1.1.1. Origin and characteristics

Staphylococcus aureus is a well-known commensal bacterium responsible for various respiratory and skin infections and one of the main causes of nosocomial infections caused by infected medical supplies. In 1883, Alexander Orgson introduced the name *Staphylococcus* and, in 1884, Friedrich Rosenbach supplied the final description of the genus *Staphylococcus* which helped the separation from the genus *Micrococcus,* also based on the relation host-pathogen, later in 1886 by Flügge [1]. It belongs to the phylum Firmicutes and the family of Staphylococcaceae being a Gram-positive bacterium with a spherical shape, denominated cocci, a slick surface, and a diameter of approximately 1µm. This microorganism is non-motile, does not form spores, it grows in clusters, short chains or pairs [2]. The cell wall is composed by a thick layer of peptidoglycans and teichoic acids composed by glycerol, sugars and/or N-acetylamino-sugars [3]. It can be aerobic and facultative anaerobic with the ability to growth in medium containing 10% of sodium chloride (NaCl) and an optimum temperature of 37°C forming yellow colonies [3]. It is coagulase-positive meaning that it is possible to clot blood plasma and it is also catalase-positive which provides resistance to hydrogen peroxide (H_2O_2), a toxic compound that is detoxified into H_2O and O_2 [1]. Both catalase and coagulase are used in clinical tests to differentiate between *Staphylococcus* and *Streptococcus* and to discriminate *S. aureus* from other *Staphylococcus* species that are coagulase negative. Additionally, it is habitually haemolytic in blood agar medium since it produces haemolysins (alpha, beta and gamma-haemolysins), and expresses several virulence factors, which helps the infection, such as proteins that promote the colonization of host tissues, toxins that can damage the tissues and proteins that can contribute to phagocytosis inhibition [2,3,4].

1.1.2. Pathogenicity, clinical manifestations, and antibiotic resistance

The pathogenesis of this bacterium can be associated with external and internal stimuli including environmental signals that lead to an activation of regulators related to gene expression and an auto induced quorum-sensing signal that responds to cell density [5]. *S. aureus* naturally produces a peptide signalling molecule (AIP – auto inducing peptide) and its accumulation activates transduction signals that, consequently, stimulates the expression of the *agr* locus which is the first accessory gene regulatory quorum sensing containing two promoters and a regulatory RNA effector (RNAIII) being one of the genes responsible for the expression of several colonizing and virulence factors[5,6,7]. When the cell density exceeds a certain amount, the AIP connects to a histidine kinase phosphorylating a response regulator that can act directly in virulence genes. RNAIII is also very important in terms of virulence with the capacity to repress or activate virulence factors like toxins [5].

S. aureus contains an extensive repertoire of virulence factors that allows its survival within the human host and avoid the immune innate system (**Figure 1.1**) [6]. To colonize the nose, which is one of the main routes of infection, this bacterium is able to adhere and invade host epithelial cells by realising various molecules termed microbial surface components recognizing adhesive matrix molecules (MSCRAMM). Relatively to the host, the lipoproteins and the peptidoglycans of the microorganism are recognised by several molecules such as host pattern recognition molecules and toll like receptors released from the infected tissues that induce the recruitment of macrophages and neutrophils [6]. To evade opsonization, *S. aureus* expresses several complement inhibitors and protein A to inactivate opsins and escape macrophages, epithelial cells, and endothelial cells. However, neutrophils are more persistent and efficient, but this bacterium developed strategies to avoid them like the secretion of chemotaxis inhibitory protein (CHIP), that prevents neutrophil recognition, and extracellular adherence protein (Eap) that inhibits neutrophil binding to intercellular adhesion molecules which leads to a prevention of leukocyte adhesion. (**Figure 1.1**) [6,8,9].

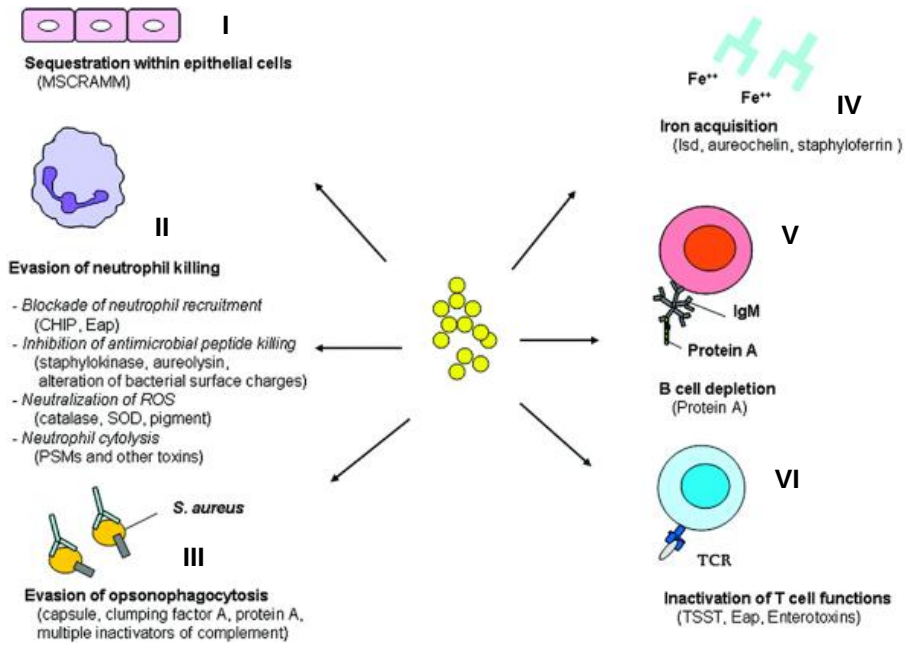

Figure 1.1 - ***S. aureus* survival strategies during infection**. (I) Microbial surface components recognizing adhesive matrix molecules, (II) Evasion of neutrophil killing with several mechanisms, (III) Evasion of opsonization with several complement inhibitors and protein A, (IV) Iron acquisition for its survival, (V) Protein A which binds to immunoglobulins to help its survival and virulence and (VI) Inactivation of T cell functions responsible for host defenses. Adapted from [6]

Besides the evasion of immune defences, the survival within the human host is dependent on nutrients acquisition and one of the most important nutrients is iron [6]. It is known that iron limitation can inhibit cellular processes and iron in abundance can be toxic due to its reactive

properties, consequently iron metabolism is essential to maintain homeostasis [5]. The existence of iron is presented, in vertebrates, in four major forms: Iron-sulphur clusters in several enzymes, extracellular molecules such as lactoferrins in the lymphoid system, transferrins present in serum intracellularly bound to ferritins and haem present in haemoglobin [5].

Siderophore-mediated acquisition and haem-iron acquisition are two essential mechanisms that *S. aureus* must counter iron deficiency. Siderophores are scavenging proteins produced by several pathogens to capture extracellular iron, bound to host glycoproteins, by removing iron from the transferrins that later is transported into the cell via ABC transporters. [5]. However, the majority of iron is confined in haem inside erythrocytes and can be obtained by hemolysins and cytotoxins responsible for the erythrocyte's lysis and haem-iron acquisition. Subsequently, haem is taken by the iron-regulated surface determinant system (Isd) which contains IsdA, IsdB, IsdC and IsdH proteins anchored to the cell wall surface, IsdD, IsdE and IsdF haem binding proteins, the IsdI and IsdG enzymes responsible for the cytoplasmic degradation and the ABC transmembrane transporters HtsABC [10]. Besides these proteins, *S. aureus* also acquires the haem regulated transporter HrtAB which protects the cells against haem toxicity [10,11].

Usually, *S. aureus* colonization is not harmful to healthy hosts and some individuals are asymptomatic nasal carriers of *S. aureus* being part of the normal flora [12]. However, it can infiltrate host defences and acquire access to deeper tissues leading to several infections which can be transmitted by direct contact of skin or tissues in the presence of lesions, contaminated objects or through the air [5]. The major infections related to *S. aureus* involves toxin-mediated diseases such as food poisoning, infected medical supplies, skin infections that can lead to bacteraemia and respiratory and gastrointestinal infections [3]. The capacity of causing several diseases also results from the ability to survive diverse stresses such as nitrosative stress as the cellular components of *S. aureus*, involved in the response to reactive nitrogen species, establishes a major defence mechanism against pathogens. The defence systems alongside nitrosative stress involves enzymes that can detoxify nitric oxide (NO) like flavohaemoglobins (Hmp), common in bacteria and yeast, able to function as NO scavengers [13,14].

The severeness of infections is associated with the ability to form biofilms creating greater susceptibility to the adherence of various surfaces giving resistance to several treatments such as antimicrobials/antibiotics [5].

Although there is plenty of knowledge related to *S. aureus* virulence, the search for a specific treatment is complex since it depends on *in vivo* signals that can inhibit or promote virulence and *in vitro* studies may differ from infection in humans [5]. Over the years, *S. aureus* acquired resistance to several antibiotics through antibiotic selection or even chromosomal mutations, but the main reason can be due to horizontal transfer of genes. The first antibiotic introduced was penicillin by Alexander Fleming around 1940 however, a few years later, penicillin resistance strains were observed in hospitals growing into a problem in the community (**Figure 1.2**) [12]. These penicillin resistance strains contain a plasmid that encoded penicillinase which is responsible for the hydrolysation of the β-lactam ring, essential for its activity [12].

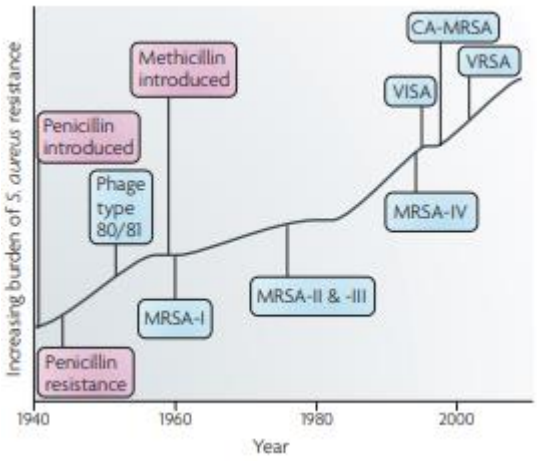

Figure 1.2 - **The four waves of antibiotic resistance in *Staphylococcus aureus*.** The relation between the years when first introduced the antibiotics until resistance strains started to appear. Adapted from [12]

Later methicillin was introduced in the market, but once again resistant strains were identified [12]. Methicillin-resistant *S. aureus* (MRSA) causes high levels of morbidity and mortality both in hospital and in the community (**Figure 1.2**) [5]. The mechanism of resistance differs from the previous antibiotic since it confers resistance to the entire β-lactam class including penicillins, cephalosporins and carbapenems and it contains a specific gene responsible for the resistance, *mecA,* which encodes the low affinity penicillin binding-protein (PBP2a or PBP2'). Outbreaks of community-associated MRSA (CA-MRSA) gave rise to several severe diseases, therefore, the signs of resistance to other antibiotics started to appear leading to more severe pathogenesis [5,12]. Vancomycin was another antibiotic to oppose the use of penicillin and methicillin but the intensive use of this antibiotic lead to vancomycin-intermediate resistant *S. aureus* strains (VISA) followed by vancomycin resistant *S. aureus* strains (VRSA) (**Figure 1.2**). However, vancomycin in combination with other antibiotics is one of the most used antibiotics to treat MRSA infections so far [12].

1.2. Tetrapyrroles

Tetrapyrroles are important pigment molecules considered one of the ancient prosthetic groups presented in all organisms [15]. They have an active role in biological processes and some of these molecules include chlorophylls, cobalamin (vitamin B_{12}), haem and sirohaem (**Figure 1.3).** Haems and chlorophylls are the most recognised tetrapyrroles for its characteristic colour, red in blood and green in plants respectively [15,16].

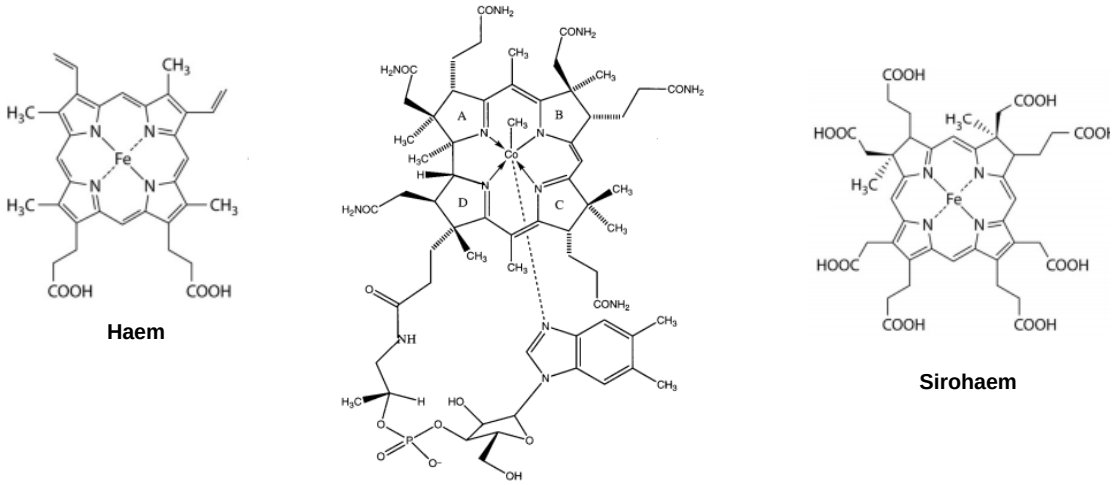

Figure 1.3 – Examples of tetrapyrroles structures. Haem, Cobalamin also known as Vitamin B_{12} and Sirohaem, respectively. Adapted from [17,18]

The structure of these molecules contains four pyrrole rings linked with methine groups creating cyclic (e.g., porphyrins) or linear (e.g., bilins) forms. Linear tetrapyrroles result from the disruption of cyclic tetrapyrroles and, on the other hand, cyclic tetrapyrroles can be divided into bacteriochlorophylls, chlorophylls, cobalamin, haems, sirohaems and others. One important role of cyclic tetrapyrroles is the ability to chelate metal ions such as Co^{2+}, an essential ion present in cobalamin that is involved in many enzymatic reactions, Ni^{2+} which is chelated by the F_{430} terminal enzyme of the methanogenesis pathway in methanogenic archaea, Fe^{2+} involved in reactions of assimilatory nitrite or sulphite reduction and Mg^{2+}, present in bacteriochlorophylls and chlorophylls that is involved in photosynthesis by trapping the sunlight and converting its energy into chemical energy [16].

1.2.1. Haem and haem biosynthesis

Haem, as one of the main focus of this thesis together with sirohaem biosynthesis, is an essential component for energy recovering electron transport chain and also a great reservoir of iron that is essential as an iron source for several bacterial pathogens[16,19,20]. It is a hydrophobic molecule that, essentially in bacteria, can be synthesised endogenously or acquired from the environment by haem uptake systems and it is involved in several important functions such as (I) cellular differentiation, (II) signalling, (III) microRNA processing and (IV) gas sensing. Other important functions relay on conserved enzymes like catalases, haemoglobin, nitric oxide synthase and others [16,19].

This compound, like other tetrapyrroles, is known to derive from the same tetrapyrrole ancestor, uroporphyrinogen III (uro'gen III) which is synthesised from δ-aminolaevulinic acid (δ-ALA) [16]. For several years the classical pathway, nowadays known as Protoporphyrin-dependent (PPD) pathway, was considered conserved in all organisms capable of producing haem. However, an alternative pathway was discovered which contains intermediates, such as sirohaem, known as Sirohaem-dependent (SHD) pathway, and another having as intermediate coproporphyrin, known as Coproporphyrin-dependent (CPD) pathway [18].

1.2.1.1. Protoporphyrin-dependent pathway

In this pathway, the formation of haem is obtained by the conversion of uro'gen III to coproporphyrinogen III (copro'gen III) via uro'gen III decarboxylase (UroD) enzyme that generates methyl groups by the decarboxylation of the acetic acid side chain [18,21]. Consequently, the copro'gen III is converted into protoporphyrinogen IX (proto'gen IX) by copro'gen III decarboxylase and it was already identified two different enzymes able to catalyse the reaction depending on the oxygen dependence: (I) oxygen dependent copro'gen III decarboxylase (CgdC) and (II) oxygen independent copro'gen III dehydrogenase (CgdH). To proceed the formation of haem, a reaction of oxidation of proto'gen IX to protoporphyrin IX involving 6 electrons can be performed by an (I) oxygen-dependent enzyme PgoX that contains a flavin adenine dinucleotide (FAD) and utilizes O_2 as terminal electron acceptor and an (II) oxygen-independent enzyme PgdH1 able to convert proto'gen IX into protoporphyrin IX in the absence of oxygen as long as an electron acceptor is present to incorporate the electrons in the respiratory chain [18] and the final step involves the incorporation of iron into protoporphyrin IX to originate protohaem, which is commonly denominated haem, catalysed by protoporphyrin ferrochelatase PpfC, a membrane associated enzyme able to utilize several metal substrates (e.g. Co^{2+}, Zn^{2+}, Ni^{2+} and Fe^{2+}) (**Figure 1.4**) [18].

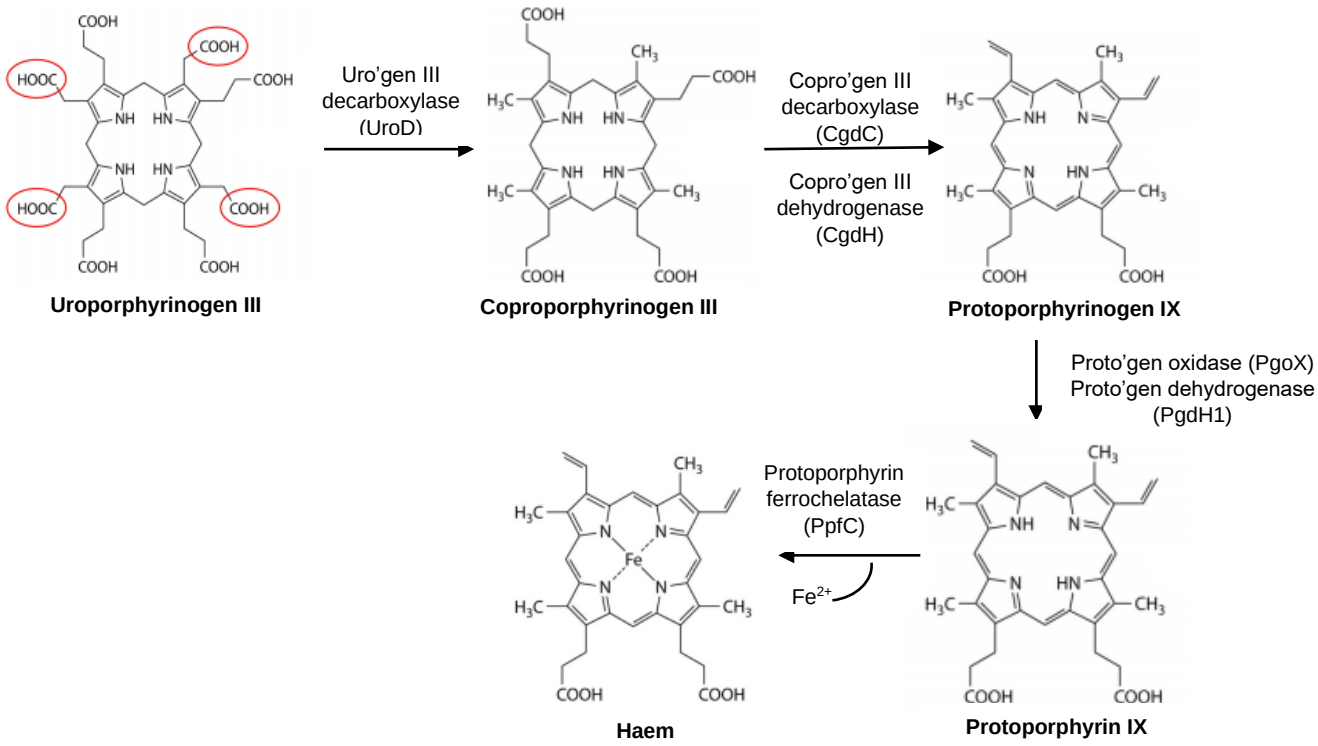

Figure 1.4 – **Protoporphyrin-dependent pathway**. Steps of the haem biosynthesis via the protoporphyrin-dependent pathway also known as the classical pathway [18].

1.2.1.2. Sirohaem-dependent pathway

This pathway, also known as the alternative pathway, initiates with a conversion of uro'gen III into sirohaem in three consecutive reaction by the multifunctional enzyme sirohaem synthase CysG: (I) methylation of uro'gen III into precorrin-2, also named dihydrosirohydrochlorin, by uro´gen III methyltransferase (UroM), (II) oxidation of precorrin-2 into sirohydrochlorin by precorrin-2 dehydrogenase (P2D) and (III) insertion of iron into sirohydrochlorin by sirohydrochlorin ferrochelatase (ShfC). The last steps involve the conversion of sirohaem by decarboxylation of the acetic acids side chains into 12,18-didecarboxysirohaem involving AhbA and AhbB proteins (alternative haem biosynthesis proteins), consequently, the acetic acid side chains are eliminated generating Fe-coproporphyrin III (or coprohaem) which is catalysed by AhbC protein. The final step is mediated by another AhbD protein which promotes the oxidative decarboxylation of Fe-coproporphyrin to generate haem (**Figure 1.5**) [18,22,23].

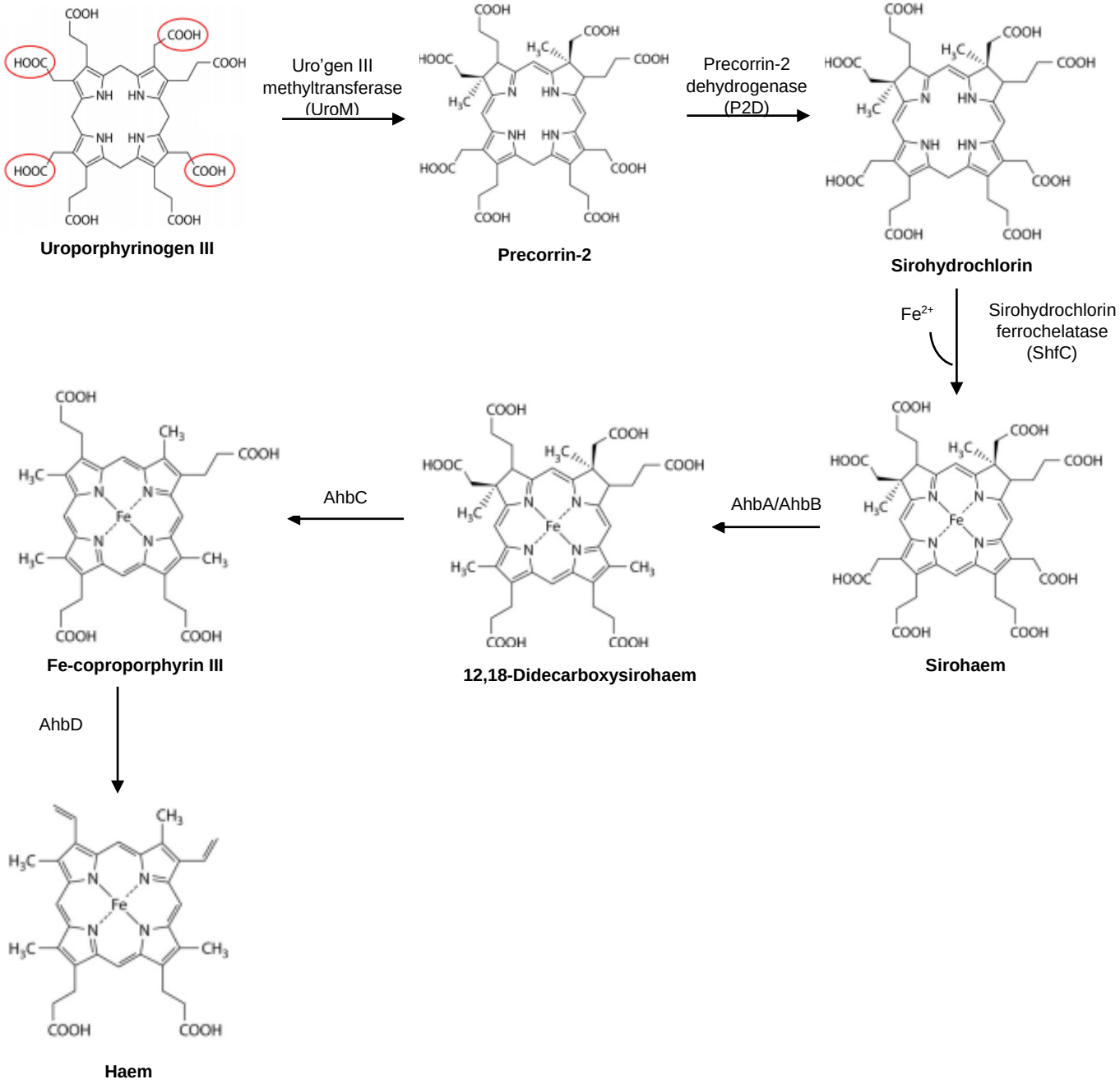

Figure 1.5 – Sirohaem-dependent pathway. Steps of the haem biosynthesis via the sirohaem-dependent pathway [13].

1.2.1.3. Coproporphyrin-dependent pathway

It was discovered that mainly Gram-positive bacteria can utilize a third different route to form haem which can be considered a transition between the Protoporphyrin-dependent pathway and the Sirohaem-dependent pathway. This new route utilizes coproporphyrin III as an

intermediate. The pathway involves four steps to convert uro'gen III into haem: (I) Uro'gen III into coproporphyrinogen III by uro'gen III decarboxylase (UroD) (II) oxidation of copro'gen III into coproporphyrin III by copro'gen III oxidase (CgoX) (III) insertion of iron to originate coprohaem by coproporphyrin ferrochelatase (CpfC), which is only present in bacteria that possess the coproporphyrin-dependent pathway, and (IV) decarboxylation of coprohaem into haem by a coprohaem decarboxylase (ChdC) (**Figure 1.6**) [18,24].

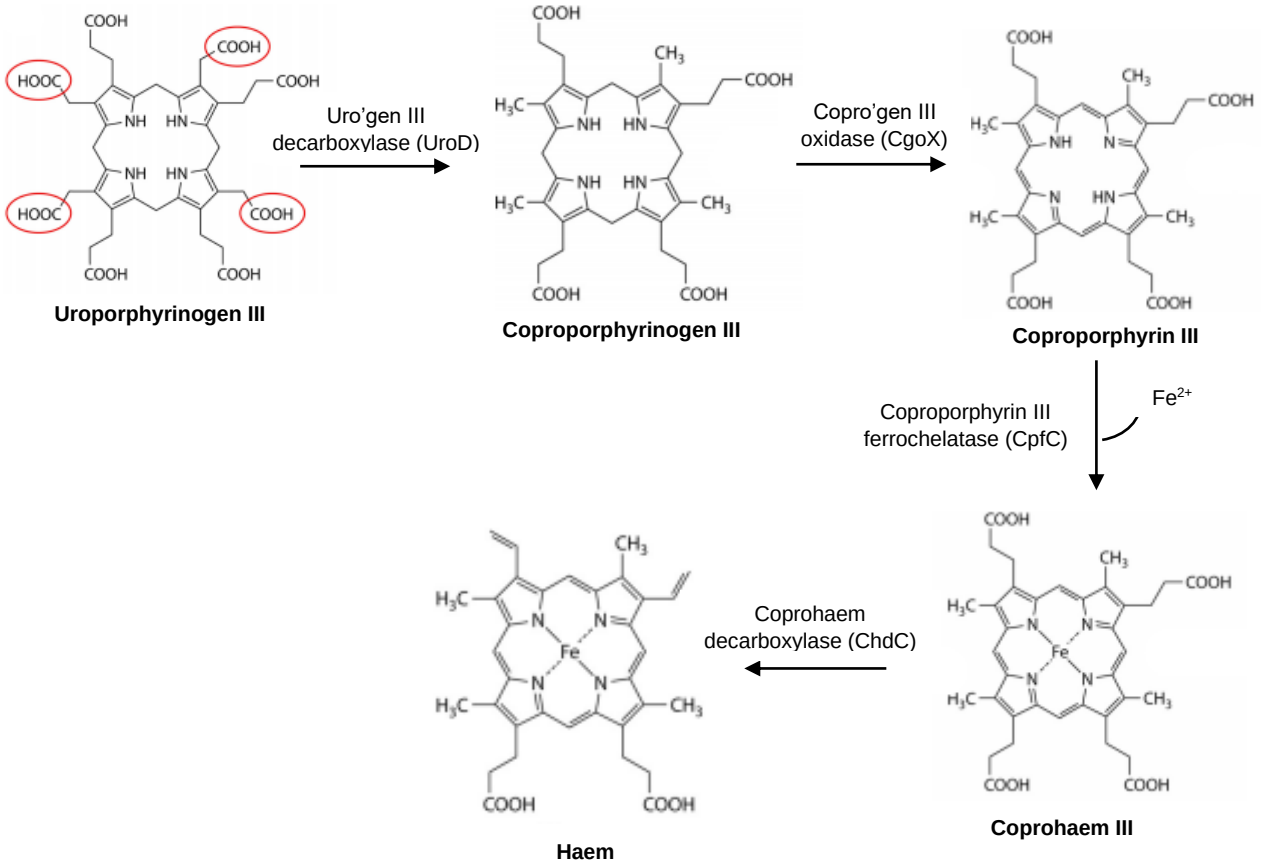

Figure 1.6 – **Coproporphyrin-dependent pathway**. Steps of the haem biosynthesis via the coproporphyrin-dependent pathway **[18].**

1.2.2. Sirohaem and sirohaem biosynthesis

Sirohaem is another iron-containing tetrapyrrole with several important functions, one of them being its involvement in sulphate and nitrate assimilation as a cofactor of nitrite reductases (NiR) and sulphite reductases (SiR) present in higher plants, prokaryotes and fungi [22,25]. The assimilation of inorganic sulphur and nitrogen depends on the accessibility of sirohaem. In the absence of sirohaem, there would be no reduced sulphur and the synthesis of cysteine and methionine [23].

As said previously, sirohaem is an important intermediate for haem biosynthesis that can be synthesised from uro'gen III by one, two or three enzymes. The multifunctional enzyme sirohaem synthase CysG converts uro'gen III into sirohaem in three consecutive reactions, which is the case of *Escherichia coli* and *Salmonella enterica,* and contains 457 amino acid residues composed by a C-terminal ($CysG^A$) with a S-adenosyl-L-methionine (SAM) dependent uro'gen III methyltransferase (SUMT) that converts uro'gen III into precorrin-2 and a N-terminal ($CysG^B$) with a precorrin-2 dehydrogenase that converts precorrin-2 into sirodydrochlorin and sirohydrochlorin ferrochelatase that provides the insertion of iron to originate sirohaem. [23,26].

In *S. aureus*, three individual enzymes are required: (I) methylation of uro'gen III into precorrin-2, also named dihydrosirohydrochlorin, by uro´gen III methyltransferase (UroM), (II) oxidation of precorrin-2 into sirohydrochlorin by precorrin-2 dehydrogenase (P2D) and (III) insertion of iron into sirohydrochlorin by sirohydrochlorin ferrochelatase (ShfC) (**Figure 1.7**) [18]. Another case of a Gram-positive bacterium that requires three individual enzymes is the *Bacillus megaterium* that synthesises sirohaem through SirA that exhibits uro'gen III methyltransferase activity, SirC with precorrin-2 dehydrogenase activity and SirB with sirohydrochlorin ferrochelatase activity [23,27].

Contrariwise, in yeast two genes are involved in the synthesis of sirohaem: *met1* and *met8*. The protein Met1p, which is the product of *met1*, contains a C-terminal similar to the CysG C-terminal with a uro'gen III methyltransferase activity and the Met8p, which is the product of *met8*, that contains a N-terminal similar to the CysG N-terminal also demonstrating a precorrin-2 dehydrogenase and sirohydrochlorin activities [23,28]. The presence of two individual enzymes, one with uro'gen III methyltransferase activities and the other with both precorrin-2 dehydrogenase and sirohydrochlorin ferrochelatase activities, has been studied since it can also occur in bacteria.

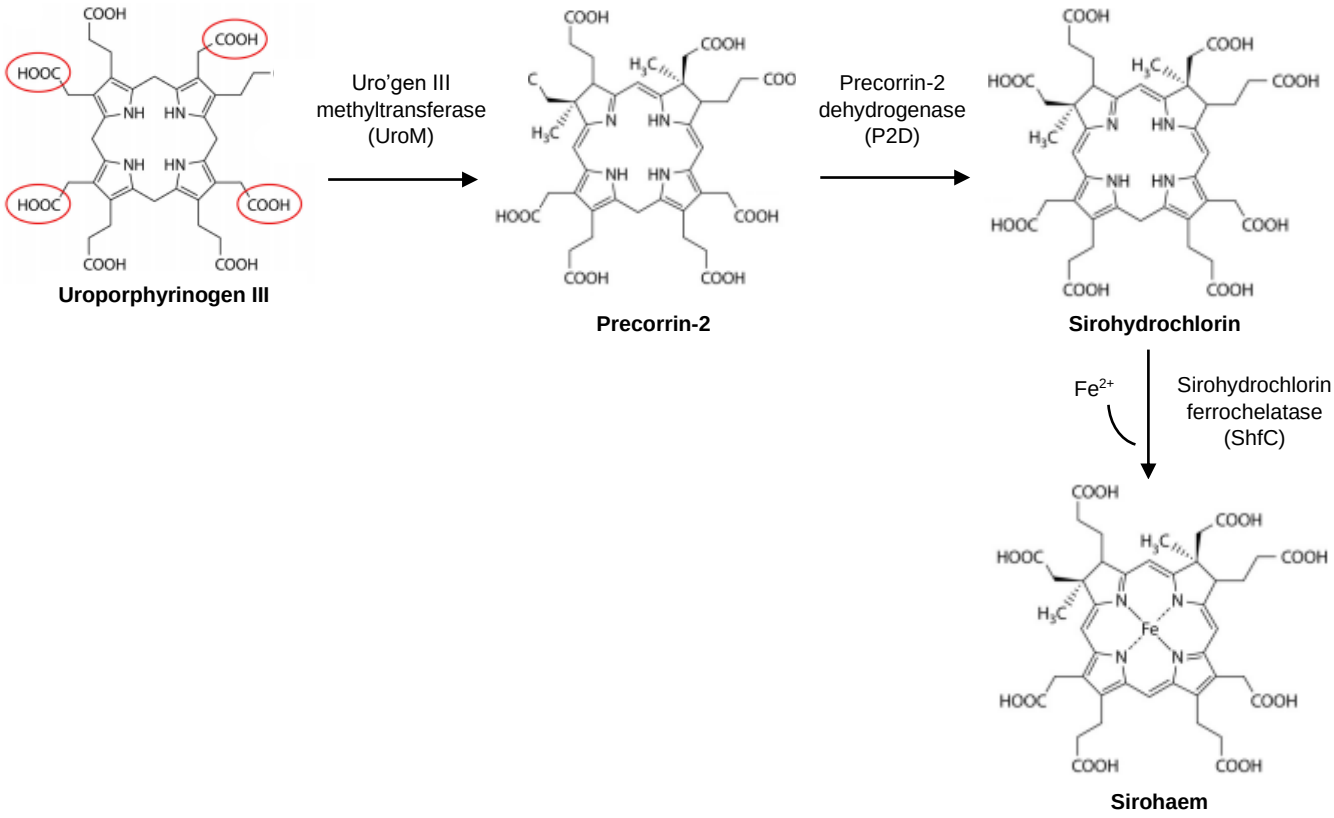

Figure 1.7 - **Steps from the sirohaem biosynthesis as part of the haem formation by sirohaem-dependent pathway**. CysG performs the three reactions: uro'gen III methyltransferase, precorrin-2 dehydrogenase and sirodydrochlorin ferrochelatase. MET1 performs uro'gen III methyltransferase and MET8 performs precorrin-2 dehydrogenase and sirodydrochlorin ferrochelatase [13].

1.3. Aim of this work

The general purpose of this work was to evaluate the function of selected genes products on haem and sirohaem biosynthesis of *S. aureus*.

For a better understanding of how we tackled these aims, this work was divided in two parts, studies related with haem biosynthesis and studies related with sirohaem biosynthesis. Therefore, the work aimed to:

1) Quantify the levels of haem when using *S. aureus* mutant strains related directly or indirectly with haem biosynthesis based on their gene function;
2) Study the effect of *S. aureus* sirohaem biosynthesis genes when complementing an *E. coli* strain unable to produce sirohaem;
3) Analyse if sirohaem biosynthesis genes have a role in the nitrate and sulphate assimilation in *S. aureus;*
4) Study the role of *S. aureus* mutant strains in sirohaem biosynthesis genes when grown under anaerobic conditions in the presence of nitrite and how it affects the survival of this bacterium.

2. Materials and Methods

2.1. Strains used in this work

For this work *E. coli* and *S. aureus* strains were used for the different experiments and are all listed in the following table (**Table 2.1**). The strains were already stored in the laboratory at -80ºC.

Table 2.1 – Bacterial strains used in this work [10,23,29].

Strains	***Designation***	***Experiment***
S. aureus		
Newman	Clinical isolate used as wild type strain	Haem quantification
NCTC	Clinical isolate used as wild type strain	
Δ*chdC* (Newman)	Coprohaem decarboxylase	
Δ*cpfC* (Newman)	Coproporphyrin III ferrochelatase	
Δ*hmp* (NCTC)	Flavohaemoglobin	
Δ*isdG* (Newman)	Iron-regulated surface determinant (Isd) proteins: Haem oxygenase	
Δ*isdC* (Newman)	Iron-regulated surface determinant (Isd) proteins: Transfer of haem across the cell wall	
Δ*hrtB* (NCTC)	Haem regulated transporter system	
Δ*htsA* (NCTC)	Transmembrane haem transporter, haem uptake and degradation	
JE2	Plasmid-cured derivative of strain LAC	Nitrate and Sulphate assimilation **/** Nitrite consumption
Δ*uroM* (JE2)	Uro'gen III methyltransferase	
Δ*p2D* (JE2)	Precorrin -2 dehydrogenase	
Δ*shfC* (JE2)	Sirohydrochlorin ferrochelatase	
Δ*nirB* (JE2)	Nitrite reductase large subunit	
Δ*nirD* (JE2)	Nitrite reductase small subunit	
E. coli		
BL21 (DE3) Gold	Recombinant protein expression strain as wild type strain	Growth and complementation assay
Δ*cysG*	Multifunctional enzyme sirohaem synthase	

2.2. Haem quantification from *S. aureus* mutants

2.2.1. Growth conditions and cell extract acquisition of the soluble fraction

The *S. aureus* strains were plated in 20ml of Tryptic Soy Agar (Difco™; TSA – pancreatic digest of casein, papaic digest of soybean, sodium chloride, agar) with the respective antibiotics, corresponding to their resistance cassettes in order to have a selective colony growth: Δ*hmp,* Δ*isdG* and Δ*isdC* were placed in TSA with 20µL erythromycin 10mg/ml (Carl Roth), Δ*hrtB* with 20µL kanamycin 30mg/ml (Carl Roth) and Δ*htsA* with 20µL tetracycline 10mg/ml (Sigma-Aldrich). The plates containing mutants Δ*chdC* and Δ*cpfC* were supplemented only with 20µL haemin 2µM (Sigma-Aldrich) since they bind to exogenous haemin and their phenotype can be rescued [10]. The wild types NCTC and Newman were plated without antibiotics. The colonies were grown overnight at 37ºC.

Individual colonies were picked and inoculated for pre-cultures (triplicates) in 10ml Tryptic Soy Broth (Difco ™; TSB – pancreatic digest of casein, peptic digest of soybean, dextrose, sodium chloride, dipotassium phosphate) with the respective antibiotics for 8h at 37ºC with an agitation of 150rpm. After 8h the OD_{600} was measured, and the cultures diluted in fresh TSB medium to an initial OD_{600}=0.1 in 100ml TSB in the same conditions as previously described with and without 100µL 400µM δ-aminolaevulinic acid (δ-ALA) (Sigma-Aldrich) at 37ºC with an agitation of 150rpm overnight (triplicates).

The cultures were centrifuged (8000rpm, 4ºC, 10min) and the pellets were collected and washed in 1ml 20mM potassium phosphate buffer at pH 7.4. The cells were harvested once again (8000rpm, 4ºC, 5min) and they were resuspended at the same concentration of 0.1g/ml of 20mM potassium phosphate buffer at pH 7.4 which corresponded to the minimal mass value per volume of solution obtained from all the strains. To break the cells, each cell suspension was incubated with 60µL lysostaphin 1mg/ml (Sigma-Aldrich) for 1h at 37ºC, and 20µL DNAse 50mg/ml (Roche) was added for 10min at room temperature (RT) to cleave the DNA. Unbroken cells were separated from the soluble fraction by centrifugation (13000rpm, 4ºC, 10min).

Total protein quantification was also done with the cell extract from all the strains (triplicates) with the BCA Protein assay (Pierce ™ – Thermo Fisher Scientific), a colorimetric method that exhibits a change of colour depending on the protein concentration. This method combines the reduction of Cu^{2+} to Cu^{1+} and a sensitive colorimetric detection of Cu^{1+} by bicinchoninic acid (BCA) beginning with the formation of a coloured complex by peptides with three or more amino acid residues followed by the reaction of BCA together with Cu^{1+} presenting an intense purple coloured reaction product that exhibits a strong absorbance at 562nm with increasing protein concentrations.

Protein quantification was extrapolated from a calibration curve done with different amounts of Albumin Standard (BSA) (**Table 2.2**). The stock solution of BSA 80mg/ml was diluted to 2mg/ml (10µL stock solution with 390µL dH_2O for a final volume of 400µL).

Table 2.2 – Calibration curve for protein quantification.

Concentration BSA (mg/ml)	*BSA 2mg/ml (µL)*	*dH_2O (µL)*
0	-	30
0.1	1.5	28.5
0.2	3	27
0.4	6	24
0.6	9	21
0.8	12	18
1.2	18	12
1.6	24	6
2	30	-

A volume of 10µL of each concentration BSA and 10µL of the cell extract from all the strains (triplicates), previously diluted 1:10 (3µL cell extract with 27µL dH_2O), were placed in a 96-well plate in duplicate together with 200µL of BCA Working Reagent (WR). The WR is calculated by the following formula and prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, reagent A:B).

(nº standards + nº samples) x (2) x (200µL WR per sample) = Total volume WR required

Afterwards, the 96-well plate was covered and incubated at 37ºC for 3min and the absorbances were read at 562nm at a plate reader (Thermo Fisher Scientific).

To quantify haem two spectrophotometric methods were used using the same cell extracts: The Alkaline haematin D-575 and the Pyridine hemochrome.

2.2.2. Haem quantification through method I: Alkaline haematin D-575

The alkaline haematin D-575 is a method where the use of an alkaline solution of a non-ionic detergent, also known as AHD reagent, changes the properties of haem in terms of stability and spectrum. It creates a product designated alkaline haematin D-575, being the absorbance peak at 575nm one of the characteristic properties of this method [30].

This method was performed by mixing 100µL of the samples with 200µL of a 0.5M sodium hydroxide (NaOH) (Eka)/2.5%TritonX-100 (Sigma-Aldrich) solution (AHD reagent), the spectrum was traced in a spectrophotometer (Shimadzu UV-1700), and the absorbances were observed at 575nm. Haem quantification was extrapolated from a calibration curve done with a mixture of different amounts of haemin with the AHD reagent (**Table 2.3**). Initially, a stock solution of 2mM of haemin (0.0026g haemin with 2ml AHD reagent) was prepared and afterwards it was diluted to 100µM of haemin (100µL stock solution with 1.9ml dH_2O).

Table 2.3 – Calibration curve for haem quantification**.**

Concentration (µM)	*100µM haemin (µL)*	*AHD reagent (µL)*
0	0	300
0.1	0.3	299.7
0.2	0.6	299.4
0.5	1.5	298.5
1	3	297
2	6	294
5	15	285
10	30	270

2.2.3. Haem quantification through method II: Pyridine hemochrome

The pyridine hemochrome method is used to determine multiple components with overlapping absorption peaks. Obeying the Lambert Beer Law, different wavelengths are measured with their known extinction coefficient and the concentration can be calculated using simultaneous equations [31].

Table 2.4 – Extinction coefficients of pyridine hemochrome [31].

Wavelength (nm)	*Reduced*	*Reduced-Oxidised*
540	-	4.37
553	29.94	23.76
555	33.88	27.6
557	34.53	28.15

NaOH, Pyridine (Sigma-Aldrich) and Potassium ferricyanide ($K_3Fe(CN)_6$) (ACROS Organics) were used with this method. The pyridine acts as a ligand for haem and the potassium ferricyanide is used to ensure that all haem is oxidised. Sodium dithionite (Alfa Aesar) is also used to reduce the haem from Fe(III) to Fe(II) state [32]. The method was performed by mixing 100µL of the samples with 200µL of a 200mM NaOH/40% pyridine + 3µL 0.1M $K_3Fe(CN)_6$ solution and the oxidised spectrum was traced. The cell extract was reduced by the addition of a small amount of sodium dithionite to each one of the samples and the reduced spectrum was traced. The concentration of haem present in the samples was determined from the absorbance of the reduced samples and done by using the difference spectrum between the reduced and the oxidised samples, both using the Lambert Beer Law with the respective extinction coefficients (**Table 2.4**). The absorbances for the reduced state were measured at 553nm, 555nm and 557nm. The absorbances for the Red-Ox state were measured by the difference between 540nm (when the peak starts) and 553nm, 555nm and 557nm (the maximum absorbance of the peak).

2.3. Complementation assay in *E. coli* cells with *S. aureus* sirohaem biosynthesis genes

E. coli strains: BL21 (WT) and the mutant Δ*cysG* (described previously at Table 2.1) were used for complementation experiments, where the mutant Δ*cysG* corresponds to an *E. coli* cell without the multifunctional enzyme sirohaem synthase CysG which performs the conversion of uro'gen III into sirohaem.

2.3.1. Preparation of competent cells with $CaCl_2$

The wild type BL21 was previously prepared into competent cells and therefore it was only necessary to prepare the mutant Δ*cysG*.

A volume of 200µL of Δ*cysG* cells were inoculated in 10ml of Luria Bertani (Carl Roth; LB – tryptone, yeast extract, sodium chloride) and grown overnight at 37ºC, 150rpm. The overnight culture was re-inoculated in 25ml of LB with 250µL of the inoculum until it reached an OD_{600}=0.5.

To make the cells competent, the culture was placed on ice for 20min, centrifuged (3000rpm, 4ºC, 10min) and the pellet was collected and washed in 10ml of 0.1M calcium chloride ($CaCl_2$) (Merck). The cells were placed, once again, on ice for 20min and were harvested (13000, RT, 10min), washed in 2.5ml of 0.1M $CaCl_2$ with 15% of glycerol (Calbiochem) and preserved frozen in aliquots with liquid nitrogen and stocked at -80ºC freezer.

2.3.2. Plasmid extraction and transformation into competent cells

The *E. coli* plasmids that contained the *S. aureus* sirohaem biosynthesis genes, were previously cloned using the Link and Lock method that allows the consecutive cloning of genes by reusing the same restriction enzymes sites, that way the genes can be connected and locked in certain positions without using several restriction enzymes [29,33].

2.3.2.1. Plasmid extraction

The cells containing the plasmids with *S. aureus* genes: pET23*uroM*, pET23*p2D*, pET23*shfC,* pET23*uroMp2D* and pET23*uroMp2DshfC* and pET23 used as a negative control were grown in 10mL of LB with 10µL of ampicillin 100mg/ml (Carl Roth) at 37ºC with an agitation of 150rpm overnight. The plasmids from the overnight cultures (**Table 2.5**). were extracted by using the QIAprep spin Miniprep kit (QIAGEN) (**Supporting Information 6.1**). The cells were harvested (8000rpm, RT, 3min) and the cell lysis was done as indicated by the manufacturer. The plasmid DNA was collected (13000rpm, RT, 10min) and the supernatant was loaded into the QIAprep spin column as the instructions were followed as indicated by the manufacturer. The eluted DNA was quantified in a Nanodrop ND-1000 UV-visible spectrophotometer (Thermo Fisher Scientific).

Table 2.5 – *E. coli* plasmids with *S. aureus* sirohaem biosynthesis genes [23].

Genes	*Designation*
pET23uroM	Uro'gen III methyltransferase
pET23p2D	Precorrin-2 dehydrogenase
pET23shfC	Sirohydrochlorin ferrochelatase

2.3.2.2. Transformation into competent cells

Each plasmid was transformed by heat shock into the Δ*cysG* and the wild type BL21 competent cells. The heat shock method consists of making the cell membrane permeable using temperature variations. For the transformation 100ng of the plasmids were mixed with 100µL of the competent cells (BL21 and Δ*cysG*) and placed on ice for 30min. Then, heat shock was applied by incubating the cells on water-bath at 42ºC for 45sec and, once again, on ice for 2min. Subsequently, 900µL of LB was added to the cells and incubated for 1h at 37ºC with an agitation of 150rpm, harvested (5000rpm, RT, 5min) and resuspended in 100µL of the supernatant. The BL21 cells and the Δ*cysG* cells with each plasmid were plated in 20ml solid LB (LA; LB with Agar from Sigma) with 20µL of ampicillin and grown at 37ºC overnight.

2.3.3. Growth conditions and acquisition of the soluble fraction

Individual colonies from BL21 and Δ*cysG* plates were picked and inoculated for pre-cultures in 10ml of LB with the respective previously mentioned antibiotics and incubated overnight at 37ºC with an agitation of 150rpm. The OD_{600} was measured, and the cultures were diluted in 200ml LB to an OD_{600}=0.05 with and without 40µL of 200µM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Carl Roth) at 37ºC with an agitation of 150rpm overnight.

Slightly different changes were implanted to the growth in order to increase protein expression. After the inoculum, same as mentioned previously, the $OD6_{00}$ was measured, and the cultures were diluted in 200ml LB to an OD_{600}=0.1. When the cells reached an OD_{600}=0.6 they were incubated at 20ºC with and without IPTG overnight with an agitation of 150rpm.

To obtain the soluble fraction, the cultures were centrifuged (1000g, 4ºC, 10min) and the pellets were collected and resuspended in 3ml of 50mM Tris-HCl buffer pH 7.5 (Tris from Carl Roth and HCl from Carlo Erba). Cells were broken in a French Press at 900psi, and the soluble fraction was obtained after centrifugation (17000g, 4ºC, 30min).

2.3.4. Spectrophotometry and Mass Spectroscopy of cell lysates

Subsequently, the soluble fraction obtained from BL21 and Δ*cysG* strains was used to trace the correspondent spectrum of each *E. coli* plasmid containing the *S. aureus* sirohaem biosynthesis genes. The spectra were traced in a spectrophotometer by mixing 150µL of each soluble fraction with 150µL of 50mM Tris-HCl buffer pH7.5 and the characteristic peaks of the

products were observed at 350nm to 400nm and 400nm to 500nm. To extract those products and confirm the results obtained from the spectra, 0.6M hydrochloric acid (HCl) was added to 300µL of each sample and mixed by vortex for 10min and centrifuged (10000rpm, RT, 10min). The samples containing plasmid were sent to Mass Spectrometry where it measures the intensity as a function of the mass-to-charge ration (m/z) per sample. The samples were treated by HPLC-MS on an Ace 5 AQ column connected to an Agilant 1100 series HPLC with a diode array detector, equilibrated first with 20% of solvent B and subsequently, its concentration was increased to 100% for 50min [10]. The products were separated by applying a gradient constituted by 0.1% Trifluoroacetic acid (TFA) and acetonitrile at a flow rate of 0.2mL/min [10] and the porphyrins were detected by their spectral absorbance at 350nm.

2.4. Confirmation of *S. aureus* mutants

To be able to proceed with the experiments, the presence of the transposon mutations of *S. aureus* mutant strains Δ*uroM,* Δ*p2D,* Δ*shfC* and Δ*nirB* were confirmed by Polymerase Chain Reaction (PCR).

The *S. aureus* strains were plated in 20ml of TSA with erythromycin 10mg/ml and the colonies were grown overnight at 37ºC. Individual colonies were picked and resuspended in 20µL of 50mM Tris-HCl buffer pH 8. To break the cells, each suspension was incubated with 1.5µL of lysostaphin 1mg/ml for 30min at 37ºC and 170µL of H_2O MilliQ was added for 15min at boiling water. Unbroken cells were separated from the soluble fraction by centrifugation (10000rpm, 5min).

Amplification of DNA sequences was achieved by PCR with the *Taq* DNA polymerase (New England Biolabs) and two specific primers, named Buster and Upstream, to verify the location of the transposon insertions used in conjunction with a primer within the gene of interest to generate a PCR product (**Table 2.6**). The primers Buster and Upstream depend on the transposon orientation within the gene and to determine the product size it must be taken into account the distance from the annealing site of the primer within the gene to the whole genome insertion site (In accordance with the Functional Genomics explorer from University of Nebraska Medical centre).

Table 2.6 – Primer sequence used for fragments amplification.

Primer	***Sequence (5' → 3')***
Buster	GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
Upstream	CTCGATTCTATTAACAAGGG
NWMN_2298_NHis_Nhe_fw	GAGCTAGCCACCACCACCACCACCACATGTATGTAGAGGAATATG
NW2298_STOP_SpeHind_Rev	CAAAGCTTTTTACTAGTTTAGTGACATAACACTGTATTAG
NW2517_CtagNheI_fw	AAGGAGCTAGCTCAACATGAATATGCCATTAATG
NW2517_STOP_SpeHind_Rev	CAAAGCTTCCGACTAGTTTATCTTACATCCAACCACGC
NW2301_NHis_Nhe_fw	CAGCTAGCCACCACCACCACCACCACGTGAATGGGAATATCATTGTTGC
NW2301_STOP_SpeXho_Rev	ATCTCGAGTTAACTAGTTTATATTTTCATTGGAATCATTAAG
Sa_nirBmiddle_Rev	CGTATGATACTTCAATACCATTTGC
hemH_pCNx_KphI_fw	TTACATGGTACCTGAAAGATAATT
hemH_pCNx_EcoRI_Rev	TCACAAGAATTCACGCTTCTTTCGTCAT

The standard PCR master mix and the PCR program used are described in the following **Tables 2.7** and **2.8,** respectively. The mix of dNTPs used are form Enzymatic.

Table 2.7 – Composition of the PCR master mix.

Component	***Volume for a 30µL reaction (µL)***	***Final concentration***
10x Standard *Taq* reaction buffer	3	1x
10Mm dNTPs	0.6	200µM
10µM primer forward/reverse	0.6	0.2µM
10µM buster/upstream	0.6	0.2µM
Template DNA	15	<1000ng
Taq DNA polymerase	0.2	1.25 units/50µL PCR
Nuclease-free water	10	-

Subsequently, the mixture was added into PCR tubes and placed in the thermocycler with the respective program (**Table 2.8**).

Table 2.8 – Standard PCR program.

Cycle step	***Temperature (ºC)***	***Time (mm:ss)***	***Cycles***
Initial denaturation	95	00:30	1
Denaturation	95	00:30	30
Annealing	50-55	00:45	
Extension	68	1min/kb	
Final extension	68	05:00	1

Electrophoresis of DNA samples was performed in 20mL of agarose gel 1% stained with 2µL of SYBR™ Safe DNA Gel Stain (Invitrogen), 5µL of each sample were mixed with 1µL of loading buffer (QIAgen) and it was used a maker of 1kb (Promega) to compare the results.

Electrophoresis was performed in TAE buffer 1x (Tris-Acetate-EDTA) at 100V for 30min using a Bio-Rad PowerPacTM 300 power supply. To visualize the results, the gel was observed on an UV transilluminator (Biorad- Geldoc XR).

2.5. Nitrate and sulphate assimilation by *S. aureus*

For this assay, several *S. aureus* strains were used: JE2 (WT), Δ*shfC,* Δ*p2D,* Δ*nirB* and Δ*nirD,* (previously described at Table 2.1).

2.5.1. Growth conditions and nitrate and sulphate assimilation

The *S. aureus* strains were plated in 20ml of TSA with the respective antibiotics, corresponding to their resistance cassettes in order to have a selective colony growth: Δ*shfC,* Δ*p2D,* Δ*nirB* and Δ*nirD* with erythromycin 10mg/ml and the wild type JE2 was plated with no antibiotics. The colonies were grown overnight at 37ºC.

Individual colonies were picked and mixed with 1ml of Phosphate Buffered Saline 1x (PBS) and were diluted to an OD_{600}=0.1. Four serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were made by mixing 90µL of PBS with 10µL of the previous dilution, consecutively for each *S. aureus* strain.

Dots of 5µL for each serial dilution correspondent to each *S. aureus* strains were added to plates. Each plate contained 20ml of M9 medium with 1mM of tiamine, 0.05mM of nicotinic acid and 1% of casamino acid, all from Sigma-Aldrich (**Table 2.9**) combined with 20µL of a different nitrogen and sulphur source: nitrate (Merck), nitrite (Merck), sulphate (Sigma-Aldrich), sulphite (Alfa Aesar), glutamine (Sigma-Aldrich) and methionine (Sigma-Aldrich) (**Table 2.10**). The plates were incubated overnight at 37ºC.

Table 2.9 – Plates composition with M9 medium.

Components	***Volume***
M9 medium	
M9 salt	4ml
Glucose ($C_6H_{12}O_6$)	400µL
Magnesium sulfate ($MgSO_4$)	40 µL
Calcium chloride ($CaCl_2$)	2 µL
Agar	10ml
Supplementation	
1µM Tiamine	20 µL
0.05µM Nicotinic acid	5 µL
1% Casamino acid	1ml

Table 2.10 – Nitrogen and Sulphur sources.

Nitrogen and Sulphur sources	***Concentration (mM)***
Nitrate	70
Nitrite	70
Nitrite	2
Nitrate + Sulphate	70 + 2
Glutamine	20
Nitrate + Methionine	70 + 1
Sulphate	2
Sulphite	5

2.6. Determination of nitrite consumption from *S. aureus* mutants: Greiss method

For this assay, the *S. aureus* strains JE2 (WT), Δ*uroM*, Δ*p2D,* Δ*shfC* and Δ*nirB* were used.

2.6.1. Growth conditions and nitrite reductase activity

Individual colonies from the plates previously prepared, were picked and inoculated for pre-cultures in 10ml of TSB with the respective previously mentioned antibiotics and incubated overnight at 37ºC with an agitation of 150rpm. The OD_{600} was measured, and the cultures were diluted in 50ml TSB, with the respective antibiotics, to an OD_{600}=0.1 supplemented with 100µL of 2mM $NaNO_2$ in a complete anaerobic atmosphere, purged of the media with N_2 for 15 minutes. The growth was maintained for 7h with an agitation of 150rpm at 37ºC, the OD_{600} was measured every hour, the cells were harvested and placed on ice.

The consumption of nitrite was extrapolated from a calibration curve done with a mixture of different amounts of $NaNO_2$ with TSB (**Table 2.11**). Initially, a stock solution of 25mM of $NaNO_2$ (0.0173g $NaNO_2$ with 10ml H_2O MilliQ) was prepared and afterwards diluted to 50µM. From this stock several dilutions of $NaNO_2$ were prepared and used in the calibration curve.

Table 2.11 – Calibration curve for nitrite consumption

Concentration (µM)	***50µM $NaNO_2$ (µL)***	***TSB (µL)***
0	0	500
5	50	450
7.5	75	425
10	100	400
15	150	350
17.5	175	325
20	200	300
35	300	150

A volume of 100µL from each strain cells of every hour collected and 100µL of the standards were placed in a 96-well plate in duplicate together with 100µL of Greiss solution (**Table 2.12**) and the absorbances were read at 540nm at a plate reader.

Table 2.12 – Greiss solution composition

Reagent	***Final concentration (m/v)***	***30mL solution in H_2O MiliQ***
Sulfanilamide (Sigma)	1%	0.3g
Naphthylene diamine dihydrochloride (Sigma)	0.1%	0.03g
Phosporic acid (H_3PO_4) (Merck)	2%	706µL

3. Results and Discussion

Several studies showed that haem and sirohaem can be related to pathogenicity and are important molecules for bacterial growth and survival [19]. The sirohaem is especially important for growth under low O_2 availability since it is the cofactor of nitrite and sulphite reductases. Therefore, we thought to investigate the role of iron-containing tetrapyrroles haem and sirohaem of *S. aureus* and the results obtained were divided in two separate sections for a better understanding.

3.1. Haem biosynthesis

3.1.1. Association between the deletion of genes involved in haem biosynthesis and their involvement in the formation of haem in *S. aureus*

Haem is an important tetrapyrrole since it contains many cellular functions as well as it is crucial for the pathogenicity. To understand how the absence of specific genes can affect the biosynthesis of haem, two haem quantification methods were used to quantify the haem present in different *S. aureus* strains. These strains were NCTC and Newman wild type strains and mutant strains namely: Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC) (described previously at Table 2.1).

The CpfC and ChdC proteins are related with the formation of haem since they correspond to the last two genes in the coproporphyrin-dependent pathway [18,29]. The IsdG and IsdC proteins are both related to Iron-regulated surface determinant (Isd) proteins. The Isd proteins capture haem from haemoglobin and transfer it across the cell wall, delivering it into the cytoplasm, where haem oxygenases release iron [10]. More recently it was shown that the *S. aureus* IsdG protein interacts with the CpfC protein of the haem biosynthesis pathway resulting in an inhibition of iron coproporphyrin chelatase activity [10]. The data obtained from previous studies indicated that strains containing IsdG have lower amounts of intracellular haem [10] and this protein also has the ability to decrease the iron coproporphyrin chelatase activity of *S. aureus* CpfC protein [10].

The Δ*hmp* mutant was included in this study to investigate the possible correlation between the *hmp* gene and the biosynthesis of haem since Hmp it is a haem containing protein responsible for the detoxification of nitric oxide and acts as a NO scavenger [13,14,34]. HtsA protein together with the HtsBC is involved in transmembrane haem transport [35] while the HrtB protein is an efflux pump that exports haem from the cytoplasm to the external environment and, together with the HrtA protein alleviates potential haem toxicity and protects the cell against outcomes of haem accumulation [36].

To quantify the amount of haem within the cells, *S. aureus* strains were grown with and without 400μM of δ-ALA to induce the formation of haem in order to observe the differences that result from the stimulation of haem biosynthesis. After the cells were broken and separated from

the soluble fraction the haem was quantified by two different methods: alkaline haematin D-575 and pyridine hemochrome method.

The haem was quantified first using the alkaline haematin D-575 method (**Figure 3.1** and **Figure 3.2**). This method uses an alkaline solution, also known as AHD reagent, constituted by NaOH and TritonX-100. This solution can change the properties of haem in terms of stability and spectrum creating a product that is able to be detected at 575nm. The calibration curves obtained throughout this assay are included in the **Supporting Information 6.2.**

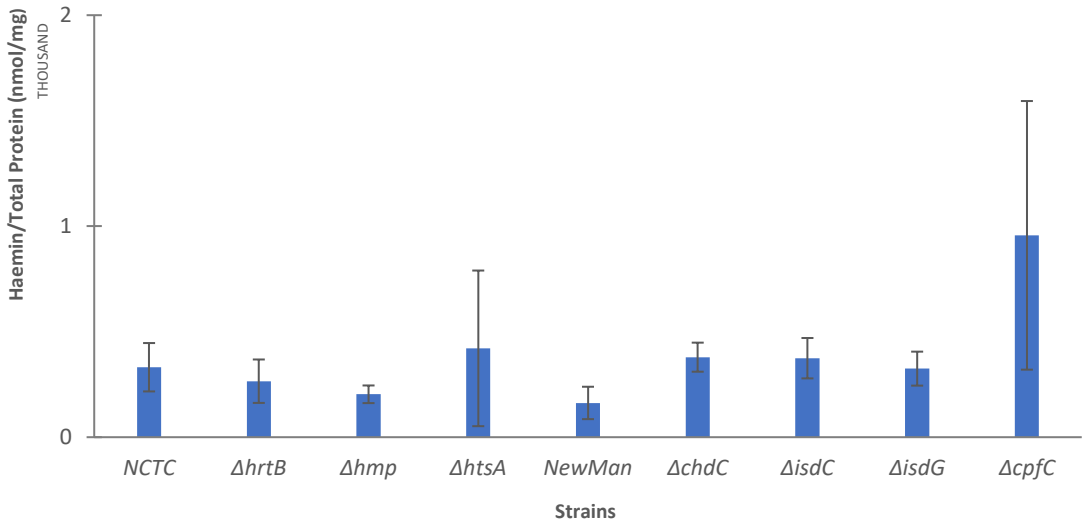

Figure 3.1 – **Haem quantification of *S. aureus* strains by alkaline haematin D-575 method**. The results correspond to the average and the standard deviation of the values obtained. Wild type strains NCTC and Newman and mutant strains Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC).

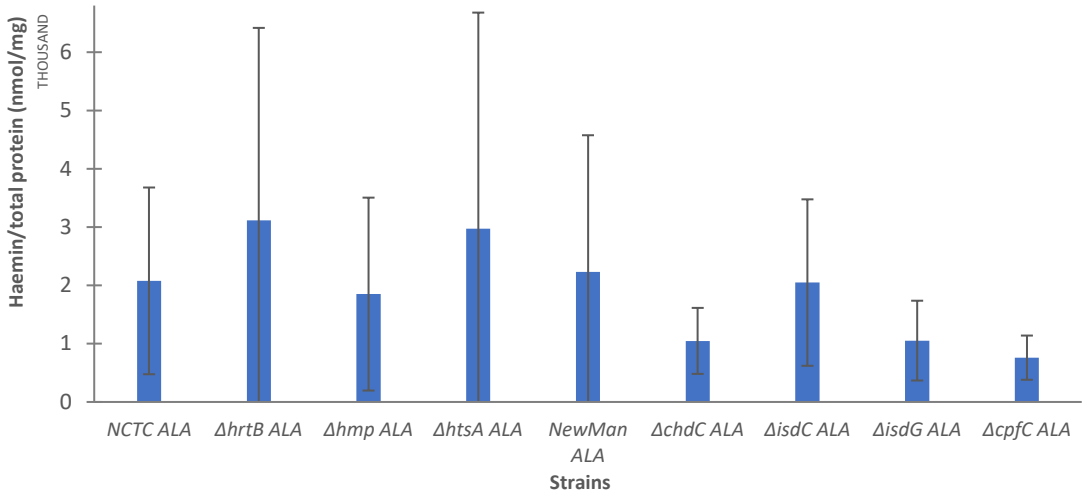

Figure 3.2 – **Haem quantification of *S. aureus* strains by alkaline haematin D-575 method in the presence of δ-ALA**. The results correspond to the average and standard deviation. Wild type strains NCTC and Newman and mutant strains Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC).

The addition of δ-ALA increases the haem content of the cells, however the variation between replicate samples also increases, leading to inconclusive results. Wild-type Newman showed values lower than the mutants and with the addition of δ-ALA, the values increased (**Figure 3.2**) however, they were similar with some mutants which may indicate that there have been problems with the wild type. The Δ*cpfC and* Δ*chdC* mutants are related with the formation of haem since they correspond to the last two genes in the coproporphyrin-dependent pathway, therefore these genes can be used directly to analyse the haem synthesis [18,29]. When comparing the Δ*cpfC* and Δ*chdC* mutants to the wild type Newman, it is possible to observe that the values of haem obtained without δ-ALA were very similar and the Δ*cpfC* mutant showed higher values (**Figure 3.1**) however, it was expected that no or very few amount of haem was formed and not as similar as the wild-type [29]. Based on the results obtained from the Δ*isdC* when compared to the wild type Newman it is possible to observe that the values are very similar indicating that this mutant does not interfere much with the formation of haem (**Figure 3.1**). In its turn, the Δ*isdG* mutant in the presence of δ-ALA showed certain similarities in value with the Δ*cpfC* mutant however, it was expected to observe higher values of haem with the Δ*isdG* mutant compared to the Δ*cpfC* mutant (**Figure 3.1** and **Figure 3.2**), as demonstrated in previous investigations, the IsdG protein interferes with the CpfC protein[10].

When compared to the wild type NCTC, the values obtained for the Δ*hmp* mutant are slightly lower but with the presence of δ-ALA are very similar (**Figure 3.2**) so it is not possible to correlate the Hmp protein to haem biosynthesis. Likewise, the values of haem obtained for the Δ*htsA* and the Δ*hrtB* mutants with the presence of δ-ALA are very similar between these them (**Figure 3.2**) and when compared to the NCTC, the Δ*htsA* mutant presents higher values and the Δ*hrtB* mutant presents similar values (**Figure 3.1**).

There may be a relation between the high variation that is observed and the use of δ-ALA, since the addiction of this substrate can generate an accumulation of porphyrins that absorb in the same wavelength as the products formed in the reaction and therefore interfere with the results.

To validate the relation of utilizing δ-ALA and since the values have high variation, another method was tested, the pyridine hemochrome. This method uses a different principle from the alkaline Haematin D-575, since it uses overlapping absorption peaks and measures different wavelengths by the Lambert Beer Law with their known extinction coefficient, as well as calculates the concentration using simultaneous equations.

This method uses NaOH and pyridine together with potassium ferricyanide ($K_3Fe(CN)_6$) that ensures that all haem present in the samples is oxidised. After the oxidised spectrum was traced, a small amount of sodium dithionite was added to reduce the haem from Fe(III) to Fe(II) state and the reduced spectrum was traced. The concentrations of haem present in the samples were determined by using the Lambert Beer Law with the absorbance of the reduced samples and by using the difference spectrum between the reduced and the oxidised samples.

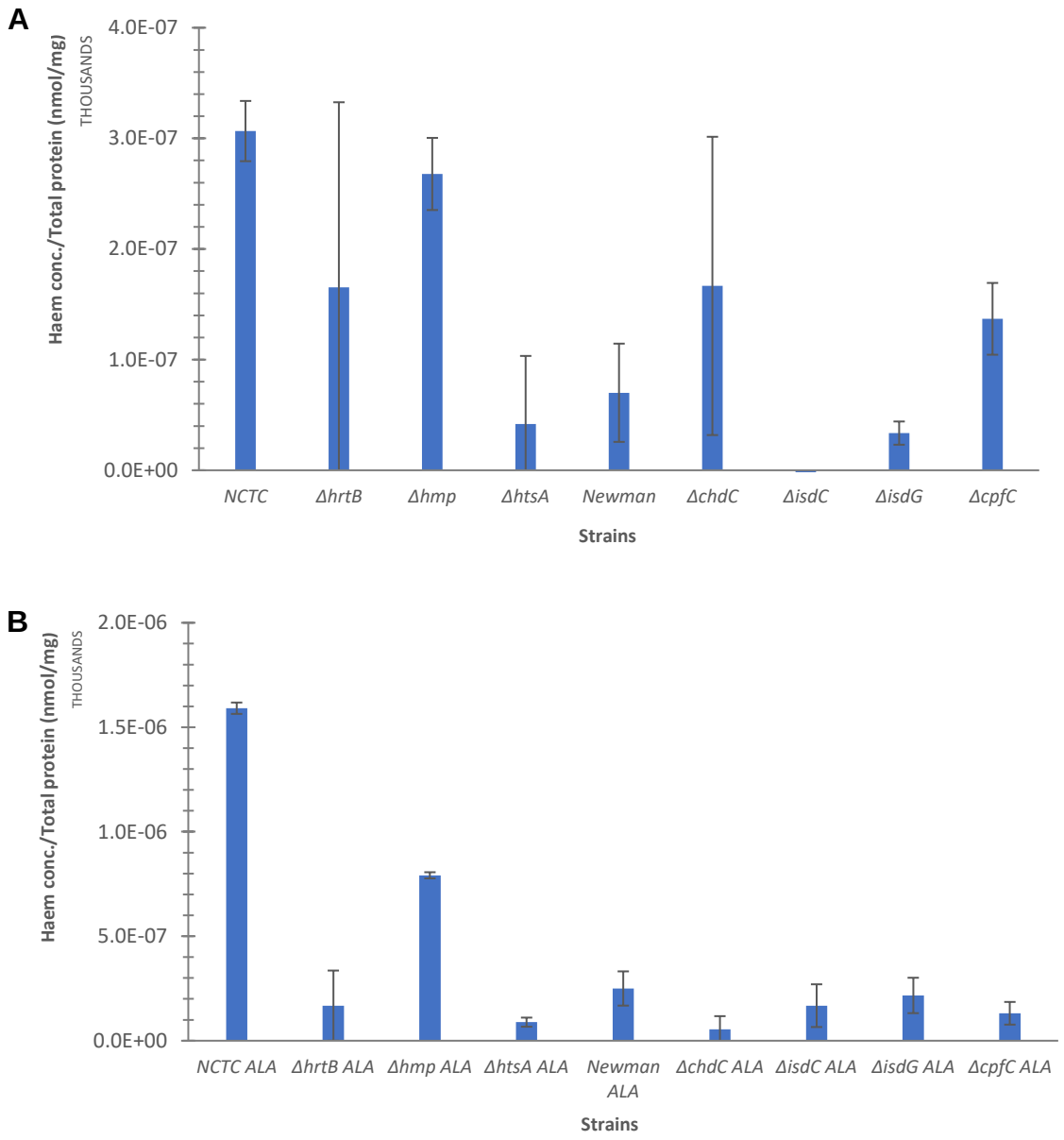

Figure 3.3 – Haem quantification of *S. aureus* strains by pyridine hemochrome method. The results correspond to the average and standard deviation of the values obtained in the reduced form at 553nm (A) and the same values obtained in the presence of δ-ALA (B). Wild type strains NCTC and Newman and mutant strains Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC).

As we could observe, the values obtained with the pyridine hemochrome method are much lower compared to the results with the alkaline haematin D-575 method. Once again, the addition of δ-ALA increases the haem content of the cells, however the variation between replicate samples also increases. When compared the values of the wild type NCTC and the Δ*hmp* mutant, it is possible to observe that the haem content is very similar when there is no addiction of δ-ALA (**Figure 3.3 A**) however, in the presence of δ-ALA there is a significant difference between them since the wild type NCTC increases its value (**Figure 3.3 B**). This result differs from the one

obtained with the alkaline haematin D-575 method (**Figure 3.1** and **Figure 3.2**) which may indicate that there is a possible correlation between the Hmp protein to the haem biosynthesis. The difference in value of haem levels obtained with the Δ*hmp* mutant, even if those differences are also among the methods used, may indicate a possible role of the Hmp protein in the regulation of haem levels however, more studies are needed to confirm this result and to understand how the Hmp protein can interfere with haem. The values of haem obtained from the Δ*hrtB* mutant and the Δ*htsA* mutant with and without the presence of δ-ALA are very similar to themselves, however, when compared to the wild type NCTC in the presence of δ-ALA, the values are lower and since the NCTC values are very high (**Figure 3.3 B**) it is difficult to comprehend the correlation between them.

With this method, the wild type Newman showed, once again, lower values than certain mutants (**Figure 3.3 A**) and with the addition of δ-ALA the values increased, however, they were very similar with some mutants (**Figure 3.3 B**). When comparing the Δ*cpfC* and Δ*chdC* mutants to the wild type Newman, it is possible to observe that the values obtained without the presence of δ-ALA (**Figure 3.3 A**) are higher than the wild type, which was not expected. In the presence of δ-ALA it is possible to observe that the wild type Newman increases its value of haem content and the mutants present lower values in comparison. Along with these results it is likely to observe the correlation between the mutants and haem biosynthesis however, the amount of haem obtained with this method is not enough to understand how these mutants interfere with haem formation since it was expected that no haem was formed or very few amounts. When comparing the values obtained from Δ*isdC* with the wild type Newman it is possible to observe that there is no value present or they are very low and almost imperceptible (**Figure 3.3 A**) however, with the addition of δ-ALA the values of haem content increase almost as the wild type Newman (**Figure 3.3 B**) and with this result it is not possible to make a conclusion. In terms of Δ*isdG* mutant, the values obtained are lower than the wild type Newman and lower than the Δ*cpfC* mutant (**Figure 3.3 A**). In the presence of δ-ALA, the Δ*isdG* mutant showed certain similarities with the wild type and higher values than the Δ*cpfC* mutant (**Figure 3.3 B**) as it was expected since the IsdG protein interferes with the CpfC protein, as demonstrated in previous investigations, decreasing the iron coproporphyrin chelatase activity of *S. aureus* CpfC protein [10].

When applying the difference between the reduced samples and the oxidised samples, it is not possible to conclude good results. Since some of the values obtained were negative or very low as well as the standard deviation is not reliable and the variation between replicate samples also increases errors (**Figure 3.4**), the values obtained of haem content present in these strains cannot be verified indicating that using the difference between the reduced and oxidised samples in the pyridine hemochrome method is not the best to observe haem content.

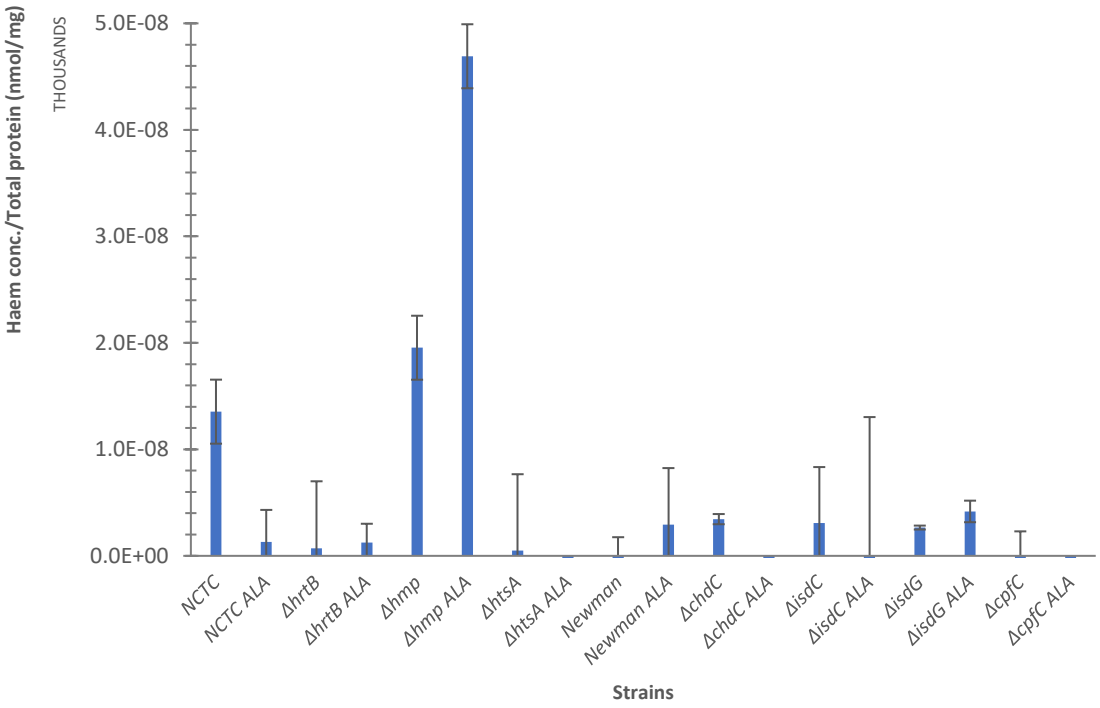

Figure 3.4 – Haem quantification of *S. aureus* strains by pyridine hemochrome method from the difference between the reduced and oxidised samples 540nm-553nm. The results correspond to the average and standard deviation of the values obtained. Wild type strains NCTC and Newman and mutant strains Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC) also in the presence of δ-ALA.

With the difference of the reduced and the oxidised samples, it was expected to be able to observe a better result of the Δ*hmp* mutant in comparison to the wild type NCTC, when compared to the alkaline haematin D-575 method and the pyridine hemochrome method at the reduced form however, these results showed values of haem in the Δ*hmp* mutant higher than the wild type NCTC. Contrary to the results obtained in the **Figure 3.3 B**, it was expected to observe a result in accordance. It was not possible to understand if there is indeed a correlation between the Hmp protein and the haem biosynthesis.

3.2. Sirohaem biosynthesis

3.2.1. Complementation assay in *E. coli* cells with *S. aureus* sirohaem biosynthesis genes

To study whether the genes of *S. aureus* were able to complement the function of an *E. coli* strain without the sirohaem synthase CysG, a complementation assay was tested using the *S. aureus* sirohaem biosynthesis genes. It was previously reported that those specific genes, *uroM, p2D* and *shfC*, work *in vitro* [23] but the function of these three genes together was never shown.

The *uroM, p2D* and *shfC*, genes used in this experiment corresponds to the genes present in the sirohaem biosynthesis of *S. aureus.* The uro'gen III methyltransferase (UroM) leads to precorrin-2, precorrin-2 dehydrogenase (P2D) forms sirohydrochlorin and sirohydrochlorin ferrochelatase forms the final product of the biosynthesis, sirohaem.

To test this, the plasmids that contained the *S. aureus* genes: pET23*uroM*, pET23*p2D*, pET23*shfC,* pET23*uroMp2D* and pET23*uroMp2DshfC* and pET23, used as negative control, were transformed into the BL21 and Δ*cysG* mutant cells. In an attempt to increase protein expression, two experiments were performed with slightly changes in their growth conditions. For the experiment I (**Figure 3.5**), the pre-cultures were diluted to an OD_{600}=0.05 with and without 200μM IPTG and grew at a normal temperature of 37ºC and for the experiment II (**Figure 3.5**), the pre-cultures were diluted to an OD_{600}=0.1 and when the cells reached an OD_{600}=0.6 they were incubated with and without 200μM IPTG at 20ºC.

After the cells were broken and separated from the soluble fraction, the spectrum of each *E. coli* strains correspondent plasmid was traced and the peaks that correspond to the products formed were observed in its characteristic wavelength between 350nm to 500nm.

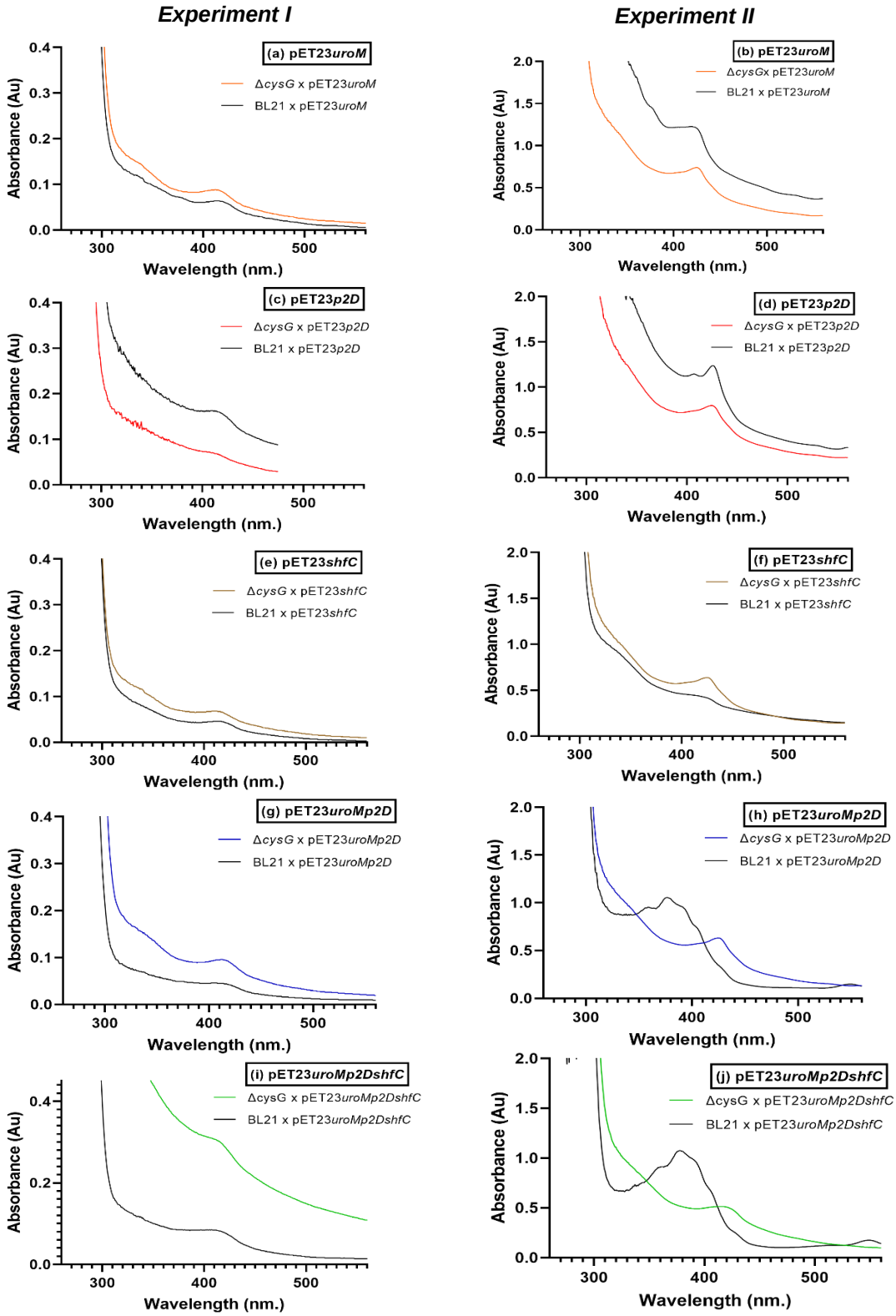

Figure 3.5 – Absorption spectra of cell extracts from *E. coli* cells BL21 and Δ*cysG* mutant with *S. aureus* sirohaem biosynthesis genes. (a, b) *E. coli* cells with plasmid containing *uroM* gene, (c,d) *E.coli* cells with plasmid containing *p2D* gene, (e,f) *E.coli* cells with plasmid containing *shfC* gene (g,h), *E.coli* cells with plasmid containing *uroMp2D* genes and (i,j) *E.coli* cells with plasmid containing *uroMp2DshfC* genes.

Most of the spectra contain a peak at around 420nm. The only exceptions are the peaks from the **Figure 3.3 h** and **Figure 3.3 j**, which represent the plasmid containing the *uroMp2D* genes and the *uroMp2DshfC* genes, respectively. We could observe a different peak at 350nm-380nm, only in experiment II where proteins were expressed at 20ºC, that could be the spectra correspondent to sirohydrochlorin and sirohaem, respectively. However, since both peaks are identical may indicate that both could represent sirohydrochlorin.

The *uroM* gene when isolated forms precorrin-2 while the genes *p2D* and *shfC* isolated should not have a function mainly in the Δ*cysG* mutant cells. In combination it can form the correspondent product as is the case of *uroMp2D* genes together forms sirohydrochlorin and *uroMp2DshfC* genes together forms sirohaem. When we compare the BL21 and the Δ*cysG* mutant cells, it was expected to see some differences between them in terms of products formed throughout the process of biosynthesis since the *E. coli* BL21 cells has the CysG protein it should be able to form sirohaem even without the plasmids containing genes nevertheless, there may have been an accumulation of products that absorb at the same wavelength, covering the results and thus it is not possible to visualize. However, when we analyse the spectra and compare between the experiments I and II, we could only see a difference and a possibility that indeed products were formed for the *E. coli* BL21 cells in experiment II since the rest showed the same peak. These differences may be derived from the change in temperature used in the experiment II to inoculate and consequently caused proteins to be more expressed. Considering the *E. coli* Δ*cysG* mutant, the protein expression may not have worked since there was not possible to use the plasmid T7 promoter as well as the leaky expression of the plasmid was not enough to be able to analyse these results.

To be able to confirm the results we sent the samples to be analysed with mass spectroscopy.

3.2.1.1. Mass Spectroscopy of cell lysates

To confirm the results obtained from the spectrophotometry and to detect other products, the cell lysates were analysed by Mass Spectroscopy by measuring the intensity as a function of the mass-to-charge ratio (m/z) per sample. Only the samples from the experiment II were analysed by mass spectrometry since they were the ones that presented differences that could refer to the formation of sirohydrochlorin.

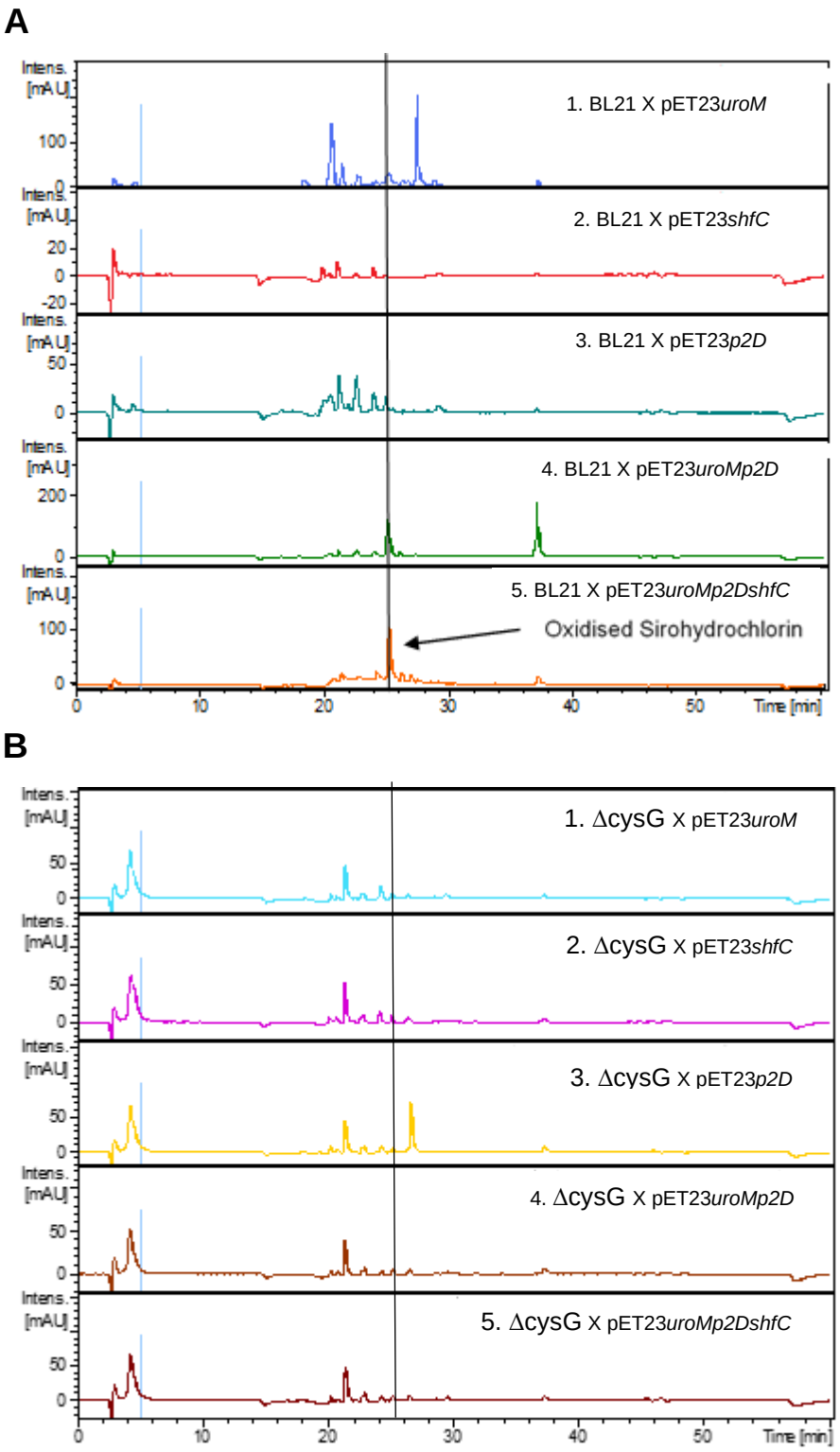

Figure 3.6 – **Protein identification obtained from HPLC-MS of cell lysates from BL21 and Δ*cysG* mutant cells.** (A) Products from BL21 cells and (B) Products from the Δ*cysG* mutant cells. The products were separated by a gradient of 0.1% TFA and acetonitrile at a flow rate of 0.2mL/min. The line represents the elution time of sirohydrochlorin.

E. coli BL21 cells expressing pET23*uroM* (**Figure 3.6 A.1**) does not show a characteristic peak since the expected product was precorrin-2, the intermediate that come from the methylation of uro'gen III however, the chromatogram shows a different peak in longer elution time that may indicate some linking. The *E. coli* BL21 cells expressing pET23*p2D* and *E. coli* BL21cells expressing pET23*shfC* (**Figure 3.6 A.2** and **Figure 3.6 A.3** respectively) do not show any peak as expected since these genes isolated should not have any function. When observed the results from the *E. coli* BL21 cells expressing pET23*uroMp2D* (**Figure 3.6 A.4**), the characteristic peak of sirohydrochlorin is easily detected, confirming the former result. In *E. coli* BL21 cells expressing pET23*uroMp2DshfC* (**Figure 3.6 A.5**) we were expecting to observe the presence of the final product of the biosynthesis, sirohaem, however the peak correspondent to sirohydrochlorin is observed, indicating that the transition from the substrate/product to *shfC* may be compromised and therefore, there is a possibility that one of the final genes present in the biosynthesis could not be working correctly in *E. coli* cells.

The **Figure 3.6 B** shows the differences between the *E. coli* BL21 cells and the results from the *E. coli* Δ*cysG* mutant cells. In the *E. coli* Δ*cysG* cells expressing pET23*uroM* (**Figure 3.6 B.1**), we were expecting to observe precorrin-2 even if in lower quantities compared to the *E. coli* BL21 cells however, it was not detected the presence of the product correspondent. As expected, the *E. coli* ΔcysG mutant cells expressing pET23*shfC* and the *E. coli* Δ*cysG* mutant cells expressing pET23*p2D* (**Figure 3.6 B.3** and **Figure 3.6 B.3**) do not present formation of product even though in the last one there is a significant peak near which may indicate some functionality related to the formation of sirohaem. The *E. coli* Δ*cysG* mutant cells expressing pET23*uroMp2D* (**Figure 3.6 B.4**) should had the presence of sirohydrochlorin even if in lower quantities compared to the *E. coli* BL21 cells. The *E. coli* Δ*cysG* mutant cells expressing pET23*uroMp2DshfC* (**Figure 3.6 B.5**) should had the presence of sirohaem even if in lower quantities. Interestingly, *E. coli* Δ*cysG* mutant cells expressing pET23*p2D* cells shows a product that is not observed in the other cell extracts indicating that the gene *p2D* may not be involved directly in sirohaem biosynthesis or can be involved in other functions with an impact in the biosynthesis however, there is not enough knowledge about this occurrence.

With the results obtained from the *E. coli* Δ*cysG* mutant cells it can be assumed that there was no complementation with the mutant and the genes from *S. aureus* sirohaem biosynthesis genes. It is only possible to observe the expression of proteins with the *E. coli* BL21 cells indicating that the *S. aureus* genes used its function to produce the respective products in the *E. coli* BL21. This *E. coli* strain did not regain its function as it was not possible to observe the products formation of each step of the biosynthesis.

3.2.2. Confirmation of *S. aureus* mutants

Before proceeding with the following experiment, the presence of the transposon mutations of *S. aureus* mutant strains Δ*uroM,* Δ*p2D,* Δ*shfC* and Δ*nirB* were confirmed by PCR.

The mutant strains were confirmed by using two specific primers Buster and Upstream in conjugation with a primer within the gene of interest. To be able to calculate the product size it must be considered the number of nucleotides added to the transposon specific primers used, which is 464bp for the Upstream and 133bp for the Buster (In accordance with the Functional Genomics Explorer form University of Nebraska Medical centre).

To be able to observe the products expected, a positive control was used to compare and to guarantee that the PCR worked well.

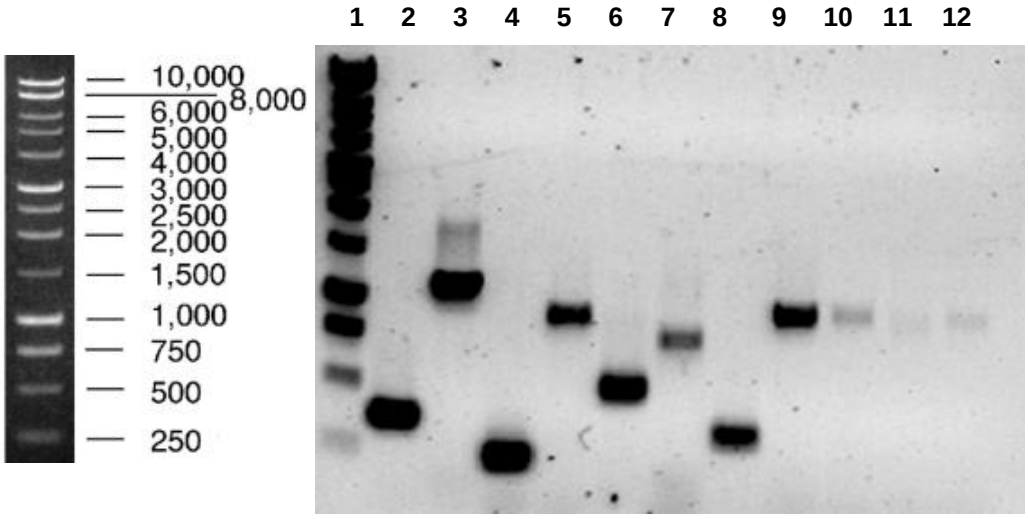

Figure 3.7 - Gel electrophoresis of the DNA products of S. aureus mutant strains from PCR. (1) 1kb Marker, (2) Δ*uroM* primer fw + buster, (3) Δ*uroM* primer rev + upstream, (4) Δ*p2D* primer rev + buster, (5) Δ*p2D* primer fw + upstream, (6) Δ*shfC* primer rev + buster, (7) Δ*shfC* primer fw + upstream, (8) Δ*nirB* primer rev + buster, (9, 10, 11 and 12) positive control for Δ*uroM,* Δ*p2D*, Δ*shfC* and Δ*nirB,* respectively.

To confirm the transposon mutation of *S. aureus* mutant strains it is necessary to find the orientation of the transposon within the gene and it must be considered the number of nucleotides for the buster and the upstream primer in accordance with the Functional Genomics Explorer, the *uroM* gene in *S. aureus* with 978bp in total is in reverse orientation and the transposon is located forward in the position 251bp within the gene. When calculated the products, it was expected to obtain as the result from the Δ*uroM* primer reverse (251bp) + primer buster (133bp) = 384bp and the Δ*uroM* primer forward (727bp) + primer upstream (464bp) = 1191bp, which can be observed in the **Figure 3.7 lane 2** and **Figure 3.7 lane 3**, respectively.

The *p2D* gene in *S. aureus* with 606bp in total is in reverse orientation and the transposon is located reverse in the position 496bp within the gene. It was expected to obtain the product

result from Δ*p2D* primer reverse (110bp) + primer buster (133bp) = 243bp and the Δ*p2D* primer forward (496bp) + primer upstream (464bp) = 960bp as observed in the **Figure 3.7 lane 4** and **Figure 3.7 lane 5**, respectively.

The *shfC* gene in *S. aureus* with 732bp in total is in reverse orientation and the transposon is located reserve in the position 344bp within the gene. The expected result from the products of Δ*shfC* primer reverse (388bp) + primer buster (133bp) = 521bp and the Δ*shfC* primer forward (344bp) + primer upstream (464bp) = 808bp as observed in the **Figure 3.7 lane 6** and **Figure 3.7 lane 7**, respectively.

Lastly, the *nirB* gene of *S. aureus* with 2406bp in total is in reverse orientation and the transposon is located reverse in the position 88bp within the gene. For this gene it was only used the primer reverse with the primer buster and the expected results for the Δ*nirB* primer reverse (88bp) + primer buster (133bp) = 221bp as observed in the **Figure 3.7 lane 8**. The *cpfC* gene was used a positive control, for every mutant strain used, since this gene corresponds to one of the last genes involved in the haem biosynthesis in *S. aureus* which is present in these mutant strains. As observed in the **Figure 3.8 lane 9, 10, 11** and **12** the products had the same number of nucleotides which indicates that the amplification of the DNA sequences was successful demonstrating that the *S. aureus* mutant strains used in this thesis were correct and that is possible to continue with the experiments.

3.2.3. Deletion of genes related to sirohaem biosynthesis impairs nitrate and sulphate assimilation by *S. aureus*

It is known that sirohaem, besides being an important intermediate for haem biosynthesis, plays an important role for nitrate and sulphate assimilation as a cofactor for nitrite and sulphite reductases [23]. Nitrate assimilation is the main process of the N-cycle and, as well as the assimilation of inorganic sulphur, depends on the accessibility of sirohaem contributing to the survival and pathogenicity of bacteria [22].

Here, the aim was to understand how lack of sirohaem biosynthesis specific genes could impact the nitrate and sulphate assimilation in *S. aureus*. For this, several *S. aureus* strains were used namely JE2(WT), Δ*p2D,* Δ*shfC,* Δ*nirB* and Δ*nirD.* The *p2D* and *shfC* genes correspond to precorrin-2 dehydrogenase and sirohydrochlorin ferrochelatase respectively, important genes present in the sirohaem biosynthesis and the *nirB* and *nirD* genes correspond to the nitrite reductase large and small subunit important for the assimilation of nitrate.

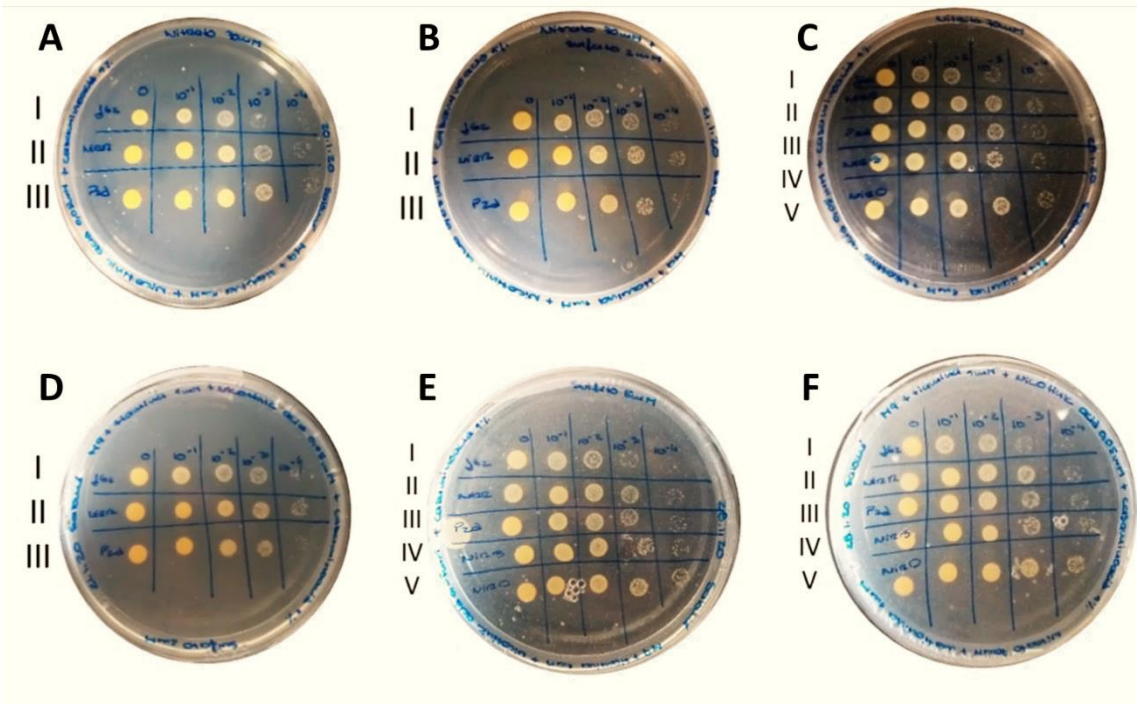

Figure 3.8 – **Nitrate and sulphate assimilation of *S. aureus* mutant strains**. All plates contained M9 medium, thiamine, nicotinic acid and casamino acid combined with different nitrogen and sulphur sources (A) Nitrate 70mM (B) Nitrate 70mM + Sulphate 2mM (C) Nitrite 70mM (D) Sulphate 2mM (E) Sulphite 5mM and (F) Nitrate 70mM + Methionine 1mM. Strains: I – JE2 (WT), II - Δ*shfC,* III - Δ*p2D,* IV - Δ*nirB*, V - Δ*nirD* all with 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions.

To access the effect of the mutations in the assimilation of nitrogen and sulphur sources the growth phenotypes were compared to the JE2 wild type strain. Consequently, the plates were grown aerobically and supplemented with different nitrogen and sulphur sources such as nitrate, nitrite and glutamine as a nitrogen source and sulphate, sulphite, and methionine as a sulphur source. Combinations of nitrate with sulphate and nitrate with methionine were also used.

The Δ*p2D and* Δ*shfC* mutants interfere with the formation of sirohaem since they are important genes for its biosynthesis and consecutively prevents the assimilation of nitrate and sulphate. The Δ*nirB* and Δ*nirD* mutants encode the large and the small subunit of nitrite reductase, respectively. Their function depends on the existence of a sirohaem cofactor to transfer electrons from NADH to nitrite to form ammonia and without these genes, *S. aureus* is unable to reduce nitrite affecting its survival and growth [23]. However, as we can observe the conditions tested did not result in different phenotypes (**Figure 3.8**). The mutants in all the plates presented the same growth even with the different nitrogen and sulphur sources. In all the plates it is possible to observe that the cell growth in wild type JE2, decreases when it is more diluted however, the mutants overcome this growth since, even with dilutions, have a longer survival rate. It is known that under anaerobic or decreased oxygen levels, *S. aureus* uses nitrate or nitrite to promote respiration as well as the genes involved in nitrate reduction are upregulated [5]. This experiment was performed under aerobic conditions and it may be what led to no assimilation by the strains

when exposed to different nitrogen and sulphur sources. To obtain better results, another experiment in anaerobically conditions was tested to understand if the mutants were required for nitrite consumption.

3.2.4. Deletion of genes related to sirohaem biosynthesis of *S. aureus* impairs its growth and ability to reduce nitrite in an anaerobic environment when supplemented with nitrite

A new assay was performed to evaluate the nitrite consumption in a medium supplemented with 2mM $NaNO_2$ under anaerobic conditions. The growth of *S. aureus* mutants Δ*nirB,* Δ*shfC,* Δ*p2D* and Δ*uroM* and the wild type JE2 was maintained for 7 hours and monitored by measuring the OD_{600} every hour. To calculate the concentration of nitrite during the growth the Greiss test was used.

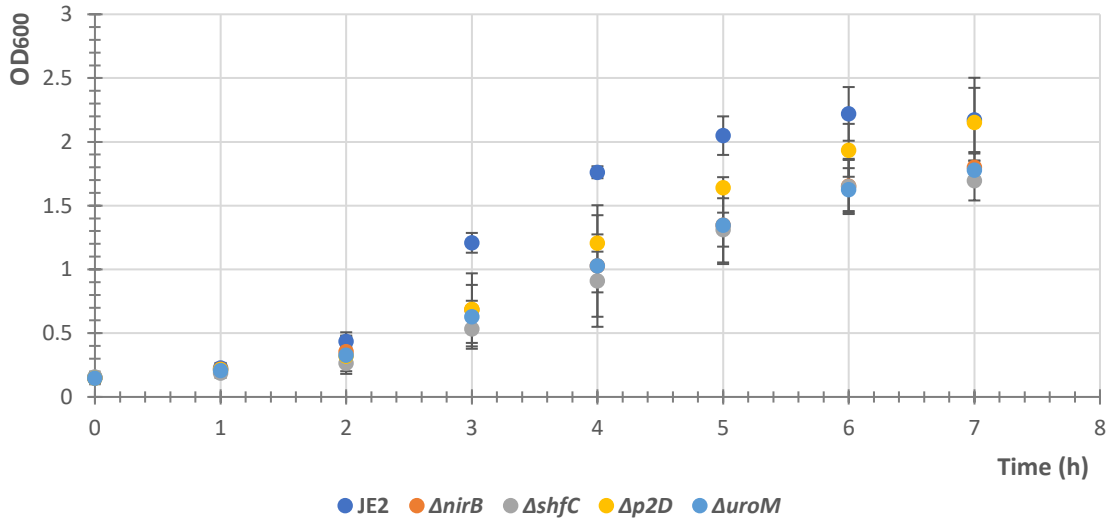

Figure 3.9 – Growth curve of S. aureus mutants. Growth measured very hour of *S. aureus* mutants and wild type strain in anaerobic conditions supplemented with 2mM NaNO2.

Figure 3.9 shows that the wild type JE2 and the mutant strain Δ*p2D* reached the same OD_{600} after 7 hours of growth, while the mutants Δ*nirB,* Δ*shfC* and Δ*uroM* demonstrated lower growth, which starts to be visible at 3-to-4-hour mark. With this result it is possible to understand how mutations can affect the growth of *S. aureus.*

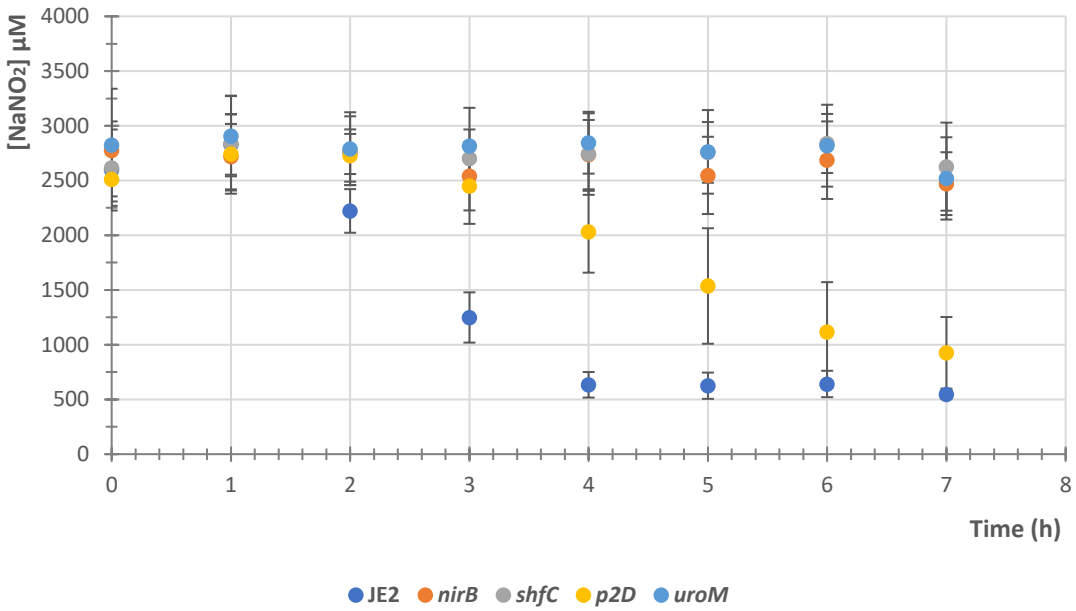

Figure 3.10 – Nitrite consumption of S. aureus mutants. Nitrite concentration during 7-hour growth of *S. aureus* mutants and wild type strain obtained from the Greiss method.

The **Figure 3.10** shows that, for a period of 7 hours, the wild type JE2 can consume almost all the nitrite available in the medium as the nitrite values decreased over time. In the medium containing the *S. aureus* mutants Δ*nirB,* Δ*shfC,* Δ*p2D* and Δ*uroM*, the nitrite present was higher. However, it can be observed that the mutants Δ*nirB,* Δ*shfC* and Δ*uroM* do not consume nitrite showing steady values of nitrite in the medium.

An interesting case of study is the mutant Δ*p2D,* capable of consuming the nitrite present in the medium even if at a slower rate than the wild type. This may indicate that the *p2D* gene may not be involved in the sirohaem biosynthesis as we know, or it can be involved in other functions. This could justify what occurred in the results from **Figure 3.6** since it was only possible to observe sirohydrochlorin and sirohaem was not formed when the genes *uroM, p2D* and *shfC* were used together. It may indicate that the transition to *shfC* could be compromised, nevertheless, more studies are needed for a better understanding.

With this experience, it is shown that the mutants Δ*nirB,* Δ*shfC* and Δ*uroM* indeed do not consume nitrite which indicates that it affects sirohaem formation in *S. aureus* as well as the nitrite reductase activity.

Tetrapyrrole Metabolism of *Staphylococcus aureus*

4. Conclusion

Staphylococcus aureus is a constitute of the human microbiota but a serious nosocomial pathogen. The pathogenicity of this bacterium can be associated with toxin-mediated diseases and several infections being a multi-resistance bacterium to antibiotics and to the host immune system, which complicates its treatment. The development of new therapeutic strategies to control those infections requires a deeper understanding of the resistance mechanisms of survival in the host.

In this work, the importance of two tetrapyrroles was analysed, haem and sirohaem, regarding the survival and pathogenicity of *S. aureus* since haem is essential for many biological processes and sirohaem, besides being involved in sulphate and nitrate reduction it can cause lesions in anaerobic environments or with low amounts of oxygen.

The results presented in this work demonstrates how the absence of specific genes can affect the haem biosynthesis and consequently, the content of haem. However, when using δ-ALA to potentiate the haem content of the cells it can generate an accumulation of porphyrins that absorb in the same wavelength interfering with the results. It was intended to investigate the possible correlation between the *hmp* gene and the biosynthesis of haem however, with the results obtained from the methods used, it is not possible to obtain data that correlates the Hmp protein to haem biosynthesis. Furthermore, new and more methods would be helpful to validate and understand how these genes are involved with haem biosynthesis and how it affects its formation.

It was possible to understand the importance of the genes involved in sirohaem production and nitrite consumption. Unfortunately, a complemented *E. coli* strain unable to produce sirohaem did not regain its function when using *S. aureus* sirohaem biosynthesis genes as it was only possible to observe sirohydrochlorin as the product from the *uroM* and *p2D* genes together in the *E. coli* BL21cells. Additionally, it was discovered that the *p2D* gene does not perform an effective role in nitrite reduction since the deletion of this gene did not affect the *S. aureus* growth, which may indicate that the *p2D* gene can be involved in other functions that are not related with the biosynthesis. This result could be related to the previous one since when the *S. aureus* genes *uroM*, *p2D and shfC* were used together in *E.* coli BlL21 cells, it was not possible to observe the final product of the reaction which is sirohaem. The transition from the susbtrate/product to *shfC* may be compromised.

This work provided knowledge to further understand the importance of haem and sirohaem present in *S. aureus.* Further studies are necessary to confirm and complement the data obtained in this work as well as *in vivo* studies to understand how haem and sirohaem can affect the pathogenicity and survival within the human host.

5. References

1. Götz, F., Bannerman, T. & Schleifer, K.-H. *The Genera Staphylococcus and Macrococcus*. *The Prokaryotes* (2006). doi:10.1007/0-387-30744-3_1

2. Gnanamani, A., Hariharan, P. & Paul-Satyaseela, M. Staphylococcus aureus: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach. in *Frontiers in Staphylococcus aureus* (2017). doi:10.5772/67338

3. Essays, I. Bergey's Manual® of Systematic Bacteriology. *Bergey's Manual® Syst. Bacteriol.* (2005). doi:10.1007/0-387-29298-5

4. Dinges, M. M., Orwin, P. M. & Schlievert, P. M. Exotoxins of Staphylococcus aureus. *Clin. Microbiol. Rev.* **13**, 16–34 (2000).

5. Balasubramanian, D., Harper, L., Shopsin, B. & Torres, V. J. Staphylococcus aureus pathogenesis in diverse host environments. *Pathog. Dis.* **75**, 1–13 (2017).

6. Cotar, A. I. Pathogenesis of Staphylococcus aureus infections. *Bacteriol. Virusol. Parazitol. Epidemiol.* **54**, 69–77 (2009).

7. Queck, S. Y. *et al.* RNAIII-Independent Target Gene Control by the agr Quorum-Sensing System: Insight into the Evolution of Virulence Regulation in Staphylococcus aureus. *Mol. Cell* **32**, 150–158 (2008).

8. De Haas, C. J. C. *et al.* Chemotaxis Inhibitory Protein of Staphylococcus aureus, a Bacterial Antiinflammatory Agent. *J. Exp. Med.* **199**, 687–695 (2004).

9. Chavakis, T. *et al.* Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat. Med.* **8**, 687–693 (2002).

10. Videira, M. A. M. *et al.* Staphylococcus aureus haem biosynthesis and acquisition pathways are linked through haem monooxygenase IsdG. *Mol. Microbiol.* **109**, 385–400 (2018).

11. Stauff, D. L. *et al.* Staphylococcus aureus HrtA Is an ATPase required for protection against heme toxicity and prevention of a transcriptional heme stress response. *J. Bacteriol.* **190**, 3588–3596 (2008).

12. Chambers, H. F. & DeLeo, F. R. Waves of resistance: Staphylococcus aureus in the antibiotic era. *Nat. Rev. Microbiol.* **7**, 629–641 (2009).

13. Gonçalves, V. L., Nobre, L. S., Vicente, J. B., Teixeira, M. & Saraiva, L. M. Flavohemoglobin requires microaerophilic conditions for nitrosative protection of Staphylococcus aureus. *FEBS Lett.* **580**, 1817–1821 (2006).

14. Nobre, L. S. *et al.* Binding of azole antibiotics to Staphylococcus aureus flavohemoglobin increases intracellular oxidative stress. *J. Bacteriol.* **192**, 1527–1533 (2010).

15. Schlicke, H. *et al.* Function of Tetrapyrroles, Regulation of Tetrapyrrole Metabolism and Methods for Analyses of Tetrapyrroles. *Procedia Chem.* **14**, 171–175 (2015).

16. Heinemann, I. U., Jahn, M. & Jahn, D. The biochemistry of heme biosynthesis. *Arch. Biochem. Biophys.* **474**, 238–251 (2008).

17. Matthews, R. G. Cobalamin-dependent methyltransferases. *Acc. Chem. Res.* **34**, 681–689 (2001).

18. Dailey, H. A. *et al.* Prokaryotic Heme Biosynthesis: Multiple Pathways to a Common Essential Product. *Microbiol. Mol. Biol. Rev.* **81**, 1–62 (2017).

19. Choby, J. E. & Skaar, E. P. Heme Synthesis and Acquisition in Bacterial Pathogens. *J. Mol. Biol.* **428**, 3408–3428 (2016).

20. Möbius, K. *et al.* Heme biosynthesis is coupled to electron transport chains for energy generation. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 10436–10441 (2010).

21. Porra, R. J. & Falk, J. E. The enzymic conversion of coproporphyrinogen 3 into protoporphyrin 9. *Biochem. J.* **90**, 69–75 (1964).

22. Tripathy, B. C., Sherameti, I. & Oelmüller, R. Siroheme: An essential component for life on earth. *Plant Signal. Behav.* **5**, 14–20 (2010).

23. Videira, M. A. M., Lobo, S. A. L., Sousa, F. L. & Saraiva, L. M. Identification of the sirohaem biosynthesis pathway in Staphylococcus aureus. *FEBS J.* (2019). doi:10.1111/febs.15091

24. Dailey, H. A., Gerdes, S., Dailey, T. A., Burch, J. S. & Phillips, J. D. Noncanonical coproporphyrin-dependent bacterial heme biosynthesis pathway that does not use protoporphyrin. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 2210–2215 (2015).

25. Neubauer, H. & Götz, F. Physiology and interaction of nitrate and nitrite reduction in Staphylococcus carnosus. *J. Bacteriol.* **178**, 2005–2009 (1996).

26. Warren, M. J. *et al.* Gene dissection demonstrates that the Escherichia coli cysG gene encodes a multifunctional protein. *Biochem. J.* **302**, 837–844 (1994).

27. Raux, E. *et al.* Identification and functional analysis of enzymes required for precorrin-2 dehydrogenation and metal ion insertion in the biosynthesis of sirohaem and cobalamin in Bacillus megaterium. *Biochem. J.* **370**, 505–516 (2003).

28. Raux, E., McVeigh, T., Peters, S. E., Leustek, T. & Warren, M. J. The role of Saccharomyces cerevisiae Met1p and Met8p in sirohaem and cobalamin biosynthesis. *Biochem. J.* **338**, 701–708 (1999).

29. Lobo, S. A. L. *et al.* Staphylococcus aureus haem biosynthesis: Characterisation of the enzymes involved in final steps of the pathway. *Mol. Microbiol.* **97**, 472–487 (2015).

30. Zander, R., Lang, W. & Wolf, H. U. Alkaline haematin D-575, a new tool for the determination of haemoglobin as an alternative to the cyanhaemiglobin method. I. description of the method. *Clin. Chim. Acta* **136**, 83–93 (1984).

31. Berry, E. A. & Trumpower, B. L. Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Anal. Biochem.* **161**, 1–15 (1987).

32. Freeman. 乳鼠心肌提取 HHS Public Access. *Physiol. Behav.* **176**, 139–148 (2018).

33. McGoldrick, H. M. *et al.* Identification and characterization of a novel vitamin B12 (cobalamin) biosynthetic enzyme (CobZ) from Rhodobacter capsulatus, containing flavin, heme, and Fe-S cofactors. *J. Biol. Chem.* **280**, 1086–1094 (2005).

34. Lewis, A. M. *et al.* Examination of the Staphylococcus aureus nitric oxide reductase (saNOR) reveals its contribution to modulating intracellular NO levels and cellular respiration. *Mol. Microbiol.* **96**, 651–669 (2015).

35. Mason, W. J. & Skaar, E. P. Assessing the contribution of heme-iron acquisition to Staphylococcus aureus pneumonia using computed tomography. *PLoS One* **4**, (2009).

36. Attia, A. S., Benson, M. A., Stauff, D. L., Torres, V. J. & Skaar, E. P. Membrane damage elicits an immunomodulatory program in Staphylococcus aureus. *PLoS Pathog.* **6**, (2010).

6. Supporting Information

6.1. Plasmid extraction through QIAprep spin Miniprep kit (QIAGEN)

The pellets from a volume of 1-5mL of an *E. coli* LB overnight cultures were collected by centrifugation (8000rpm, 3min, RT). The supernatant was discarded, and the pellet was resuspended in 250µL of Buffer P1. Afterwards, 250µL of Buffer P2 was added and mixed carefully by inverting the tube 4-6 times until the solution becomes clear and then, 350µL of Buffer N3 was added and mixed with the same process. The lysate resulting from the mixture was centrifuged (13000rpm, 10min) and the supernatant collected was transferred to a QIAprep spin column which was centrifuged (13000rpm, 30-60s) and the flow-through was discarded. The column was washed twice by adding 0.5mL of Buffer PB, centrifuged (13000rpm, 30-60s) and discarded the flow-through and the second time by adding 0.75mL of Buffer PE, centrifuged (13000rpm, 30-60s) and discarded the flow-through. The QIAprep spin column was transferred to a collection tube and centrifuged (13000rpm, 1min) to remove residual wash buffer, afterwards, to elute de pDNA 50µL of Buffer EB (10mM Tris-HCl, pH 8.5) or water was added and after 1min was centrifuged for 1min.

6.2. Haem quantification by the Alkaline haematin D-575 method

The content of haem present in the cells was extrapolated from a calibration curve with a mixture of different amounts of haemin with the AHD reagent.

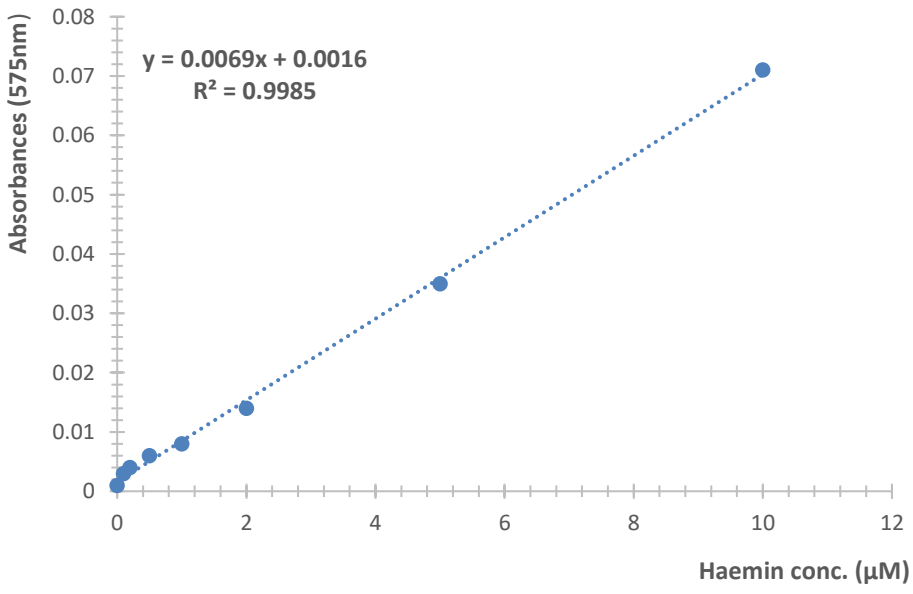

Figure 6.1 – **Calibration curve used in the alkanine haematin D-575 method**. Calibration curve correspondent to different amounts of haemin with the AHD reagent used to calculate the haemin present in *S. aureus* NCTC and Newman wild type strains and mutant cells Δ*cpfC* (Newman), Δ*chdC* (Newman), Δ*hmp* (NCTC), Δ*isdG* (Newman), Δ*isdC* (Newman), Δ*hrtB* (NCTC) and Δ*htsA* (NCTC).

To be able to quantify the haem content of the cells, total protein quantification was also performed with the BCA Protein assay. Protein quantification was extrapolated from a calibration curve with different amounts of Albumin Standard (BSA).

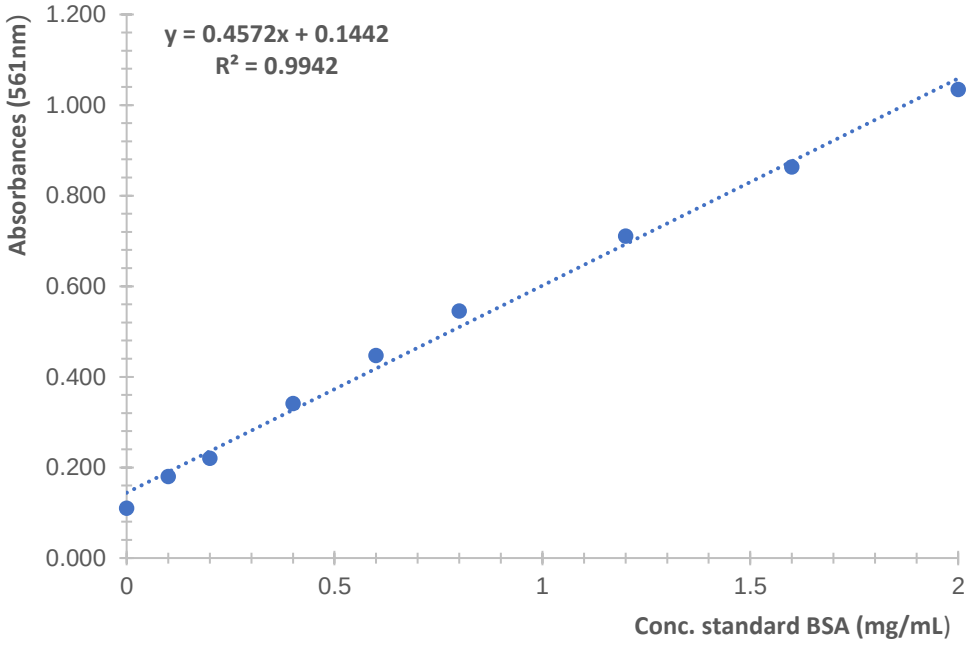

Figure 6.2 – Calibration curve for total protein quantification with the BCA Protein assay for the alkaline haematin D-575 method. Calibration curve correspondent to different amounts of Albumin Standard (BSA) up to 2mg/mL

6.3. Haem quantification by Pyridine hemochrome method

Total protein quantification, with the BCA Protein assay, was also performed for the pyridine hemochrome method. Protein quantification was extrapolated from a calibration curve with different amounts of Albumin Standard (BSA).

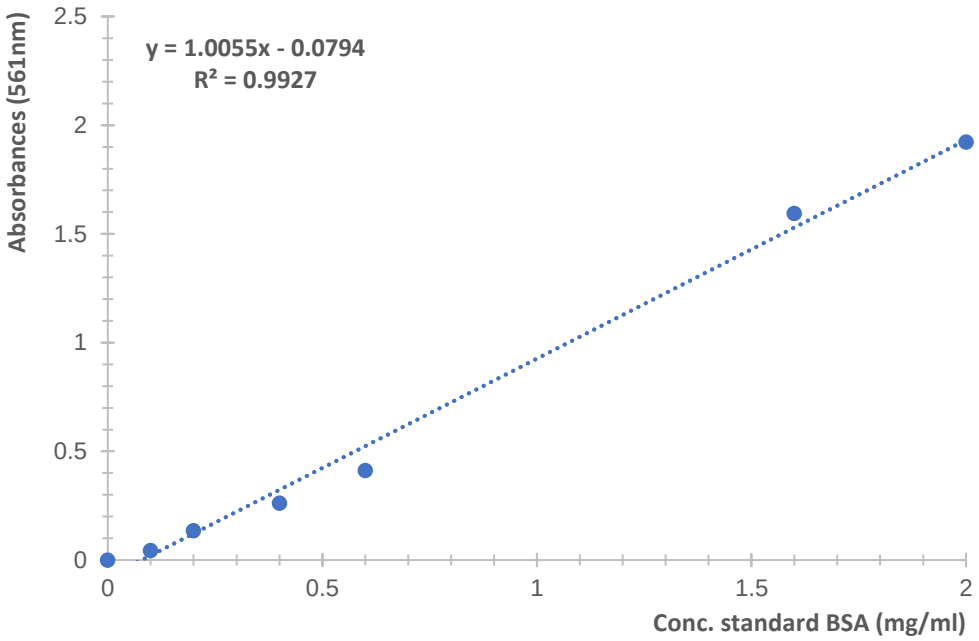

Figure 6.3 – Calibration curve for total protein quantification with the BCA Protein assay for the pyridine hemochrome method. Calibration curve correspondent to different amounts of Albumin Standard (BSA) up to 2mg/mL